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THIS ISSUE

AND THE NEXT (volume 47, numbers 1 and 2)

ARE DEDICATED TO

MERKEL HENRY JACOBS

IN HONOR OF HIS 55 YEARS

OF STUDY AND TEACHING

AT THE UNIVERSITY OF PENNSYLVANIA

AND HIS HELPFUL

SERVICE ON THE BOARD

OF EDITORS OF THE J.C.C.P.

Merkel Henry Jacobs

MERKEL HENRY JACOBS
AND THE STUDY OF CELL PERMEABILITY

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It is highly fitting that the present number of the JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY should be dedicated to Professor Merkel Henry Jacobs in the year of his retirement as Professor of Physiology at the Medical School of the University of Pennsylvania. This also marks the time of his seventieth birthday. Many of his papers have appeared in this Journal, and he has served as an associate editor since it started in 1932. Moreover, his general field of interest, cellular physiology, and his special subject for research, cell permeability, have become major branches of experimental biology. He has greatly stimulated the progress of this department of learning. Occasionally it is worthwhile for scientists to pause and look backward at the orderly development of a subject. The following brief survey of the history of cell permeability will indicate Professor Jacobs' relationship to it.

Interest in the permeability of cells to various substances began in the latter part of the nineteenth century, perhaps chiefly as a sequal to the work of Wilhelm Pfeffer and Hugo de Vries, who clarified the laws governing osmotic pressure, and laid the foundation for the birth of the new science of physical chemistry. This particular branch of physical chemistry, the theory of solutions, was almost immediately associated with the names of two illustrious chemists, J. H. van't Hoff (osmotic pressure and the gas laws, 1885) and Svanté Arrhenius (electrolytic dissociation, 1887).

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Although Carl von Naegeli and Julius von Sachs had investigated the osmotic properties of living cells in the eighteen-fifties and Moritz Traube had prepared semipermeable membranes in 1867, Pfeffer's book, "Osmotische Untersuchungen" (Leipzig, 1877), contained the quantitative data for understanding the passage of water into and out of plant cells. Pfeffer's continued interest in the subject is shown by the title of his 1890 paper, a monograph of 156 pages, "Zur Kenntnis der Plasmahaut und der Vacuolen." De Vries' contribution, "Untersuchungen über die mechanische Ursachen der Zellstreckung" (1877) was followed (1884–1885) by his papers on isotonic coefficients. The words, "plasmolysis" and "semipermeable" came into general use among physiologists, and "osmosis" and "permeability" studies became closely associated as a result of this early work.

The late eighteen-nineties were characterized by growing interest in the nature of the "plasma membrane" and the types of substances which can or cannot pass through. Research in this field was led by H. J. Hamburger and E. Overton, particularly Overton, whose lipoid theory became a byword among all who professed an interest in the subject of cell permeability, both plant and animal. G. Gryns, H. Koeppe and S. J. Hedin continued the study of blood corpuscles begun by Hamburger.

During the first two decades of the twentieth century, the names of R. Hoeber, A. Nathanson, W. Ruhland, W. W. Lepeschkin, R. S. Lillie, W. J. V. Osterhaut, J. F. McClendon, A. Tröndle, E. N. Harvey, H. Fitting, W. Stiles, S. C. Brooks, and K. Hofler, among others, were particularly associated with osmotic and permeability research. Indeed, at this time the cell physiologist appeared never actually to "penetrate" the cell and study internal mechanisms, so much time was devoted to discussion of the properties of the surface.

Professor Jacobs entered the field of cell permeability by a somewhat circuitous route — through interest in the marked and rather specific action of carbon dioxide on cells, an effect which is out of all proportion to the hydrogen ion concentra-

tion of its aqueous solutions. This peculiarity turned out to be due to the marked permeability of cells to carbonic acid molecules, which enter readily and dissociate inside, thereby producing an intracellular acidity, whereas hydrogen ions do not enter a cell except under special circumstances. The idea is presented in two of his 1920 papers, ''To what extent are the physiological effects of carbon dioxide due to hydrogen ions,'' followed by ''The production of intracellular acidity by neutral and alkaline solutions.''

The germ of the above interest can be traced to an earlier paper, ''Studies on the physiological characters of species. I. The effects of carbon dioxide on various protozoa'' ('12), which followed another publication, ''Physiological studies on the protozoan parasites of Diadema setosum'' ('11). The parasites of the sea urchin, Diadema, live in an environment where they are frequently exposed to rather high concentrations of CO_2. Study of such unfavorable environments was a logical result of Jacobs' first paper, a doctoral thesis, ''The effects of desiccation on the rotifer, Philodina roseola'' ('09). His Ph.D. degree was taken under the late Professor E. G. Conklin at the University of Pennsylvania (Zoology Department) in 1908.

It is hard to believe that such a thesis title could lead to an intensive study of the permeability of cells and particularly to permeability of the erythrocyte, but such is the case. There are a few diversions among his publications, for example, ''Acclimatization as a factor affecting the upper thermal death points of organisms'' ('19) and ''A graphic method of balancing the army ration'' ('19), by Captain Jacobs, but the trend to permeability is definite and straightforward. By 1924, Jacobs had written the chapter on ''The Permeability of Cells'' for Cowdry's ''General Cytology,'' and in 1927 delivered a Harvey Society lecture, ''The exchange of material between the erythrocyte and its surroundings.''

Since that time, the titles of Jacobs' papers and those of his students have been almost entirely in the field of cell permeability, frequently that of the erythrocyte or of the

echinoderm egg. The broad implications of the work are indicated by the paper on, "Osmotic hemolysis and zoological classification," delivered before the American Philosophical Society in 1931.

From time to time the results have been summarized in such general papers as "The permeability of the erythrocyte" in the "Ergebnisse der Biologie" for 1931 and "The measurement of cell permeability with particular reference to the erythrocyte," published in "Modern Trends in Physiology and Biochemistry" ('51). The related subject of "Diffusion Processes" in the "Ergebnisse der Biologie" for 1935 was exhaustively treated and the article might have been published as a book.

The above titles serve as an index to Jacobs' interests, but they cannot give a proper idea of his method of experimentation. This can best be described by two words — "simple" and "quantitative." Jacobs required no complicated apparatus to measure and record the swelling of cells, from which the penetration of a substance can be deduced.

In his early work, it was sufficient to place a suspension of red blood corpuscles in a test-tube before an incandescent lamp filament, to add the substance whose penetration was to be tested, and to record with a stop-watch the time for the incandescent filament to become clearly visible. This time represented a critical point in the curve of osmotic swelling of the corpuscles, when hemoglobin molecules passed through the erythrocyte membrane and the corpuscles lost their ability to scatter light. By proper mathematical treatment, the time could be interpreted in terms of penetration of the added substance. Only in later years did he bow to convention and adopt the automatic recording device.

It is interesting to note that this osmotic method of measuring permeability was the one chosen by Overton in his early study of plant cells and one used by early workers on permeability of erythrocytes — the hemolysis method. Because of the accuracy with which the hemolysis point can be determined, this method is particularly applicable to red corpuscles,

provided conditions are chosen so that hemolysis becomes a point on the swelling curve when the time will be proportional to rate of entrance of the material tested. These conditions were not always selected by the early workers and much confusion in interpretation resulted.

In his lectures, Jacobs took pains to explain clearly the pitfalls of the hemolysis method and also to emphasize the value of erythrocytes for permeability studies — that they are isolated cells which can be obtained in any quantity, in any place; by a simple prick with a needle, one always had a supply on hand to work with, no matter where one worked.

The late 1920's and early 1930's can be designated as the quantitative age of permeability research. This was the period when the laws for movement of water through cell membranes were formulated, and the equations turned out to be highly complicated.

I have always felt that Jacobs' mathematical approach to cell problems stemmed from June of 1908 when he broke a leg climbing in the little-known Selkirk Range of the Canadian Rockies and lay alone at tree-line in a pup-tent for three days, while his companion went for help. I was a member of the first rescue party, saw his leg set, and helped make him as comfortable as possible, with "Elements of Calculus" at his side. He spent the summer in this spot and was carried out to civilization in late August. For a studious person, what more favorable situation to master the complexities of higher mathematics! Jacobs later made good use of the knowledge by giving a course of lectures on mathematics suited to workers at the Marine Biological Laboratory, Woods Hole, Massachusetts, where he was Director, and of which he has been a Trustee for many years.

At Woods Hole, the sea urchin, Arbacia, is abundant, and the spherical egg-cell is unusually favorable for permeability studies. Interest in its osmotic behavior was started by R. S. Lillie in 1916 and may be said to have culminated in the work of two of Jacobs' colleagues, B. Lucké and M. McCutcheon, summarized in their Physiological Review paper,

"The living cell as an osmotic system and the permeability to water" ('32).

During the same period, Jacobs was working on the permeability of cells to specific substances. His results appeared in the first number of the Journal of Cellular and Comparative Physiology, February 20, 1932, "A simple method for the quantitative measurement of cell permeability," with Dorothy H. Stewart as co-author. This was the first of a series of papers with Dr. Stewart in which the laws of solute penetration were accurately formulated and the sea urchin egg was studied quantitatively for permeability to various nonelectrolytes. At about the same time Jacobs applied similar laws to the erythrocyte and a series of papers appeared with another student, Arthur K. Parpart, now managing editor of this Journal, and an expert, like his teacher, on the physiology of the erythrocyte. Both men have been particularly concerned with the penetration of electrolytes and the problems connected with ion exchange across the cell surface.

It is fair to say that the studies of Jacobs and his students have greatly influenced the approach to permeability problems throughout the world. Following Ruhland, an exhaustive quantitative study of permeability of plant cells to various substances was begun by R. Collander and H. Bärlund in Finland in the thirties and is continuing to the present day. Unfortunately, space prevents more detailed consideration of this work and the later experiments of many physiologists. Books on permeability have appeared from time to time, the two latest by S. C. and M. M. Brooks ('41) and by H. Davson and J. F. Danielli ('43). Symposia have been devoted to such subjects as "Ion transport across membranes" ('54) and "Active transport and secretion" ('54). Permeability research has prospered, largely as a result of Jacobs' influence. Many of the contributions have appeared in this Journal. As editor, he has spent much time reading manuscripts and given kindly advice to young authors who have submitted papers. Long may he continue in this capacity, and long may he carry on with the important research on cell permeability which he started thirty-five years ago.

A STUDY OF THE LIBERATION OF ORTHOPHOSPHATE FROM ADENOSINE TRIPHOSPHATE BY THE STROMATA OF HUMAN ERYTHROCYTES [1,2]

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FOUR FIGURES

Enzymes that liberate orthophosphate from adenosine triphosphate are widely distributed in animal and plant cells, but comparatively little is known about their exact physiological function. The present paper had its origin in the finding of Rothstein and Meier ('48) that dephosphorylation of ATP by intact yeast cells occurs at the cell surface. It was thought that if a similar condition exists in other cells, a particularly favorable form of material for its study might be the mammalian erythrocyte, which is unique in the ease and completeness with which its surface ("ghost," stroma) can be separated from its other constituents. This surmise proved to be correct.

As the work was nearing its completion, some of its conclusions were anticipated by a paper by Clarkson and Maizels ('52). In general, however, the points of view of the two investigations are entirely different and the present results for the most part do not duplicate those already published. In the

[1] This study formed part of a dissertation presented to the faculty of the Department of Physiology of the University of Pennsylvania School of Medicine in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] The major part of this work was done during the tenure of a Public Health Pre-doctorate Fellowship administered by the National Heart Institute.

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few instances where they do, appropriate mention will be made of their confirmatory or contradictory character.

MATERIAL AND METHODS

Human erythrocytes, obtained from a vein or finger of the same individual, were used exclusively in the present work. Coagulation was prevented with heparin. The blood was mixed with 10 times its volume of 0.9% NaCl and washed twice; the resulting suspension was then adjusted by an optical method (Ponder, '35) to a standard concentration of 5×10^9 cells per milliliter. Since stromata could be obtained from such cell suspensions with virtually no losses, the same figure represents the standard concentration of stromata employed in all the experiments to be described except where otherwise noted.

Stromata were prepared from a standard erythrocyte suspension by the addition of 10 times its volume of ice-cold distilled water; after standing at this temperature for 10 minutes the cell "ghosts" were reversibly agglutinated by acidification of the medium with HCl to pH 6.5 (not lower than 6.0), centrifuged at $400 \times g$ for 5 minutes, washed 3 or 4 times in 10 times their volume of ice-cold distilled water and stored in a refrigerator until needed.

In order to obtain reproducible results with such stromata it was found necessary to pay strict attention to three sources of error that are largely avoidable by the use of the technique just described. These are (1) deterioration of the enzyme with time, which is more rapid the higher the temperature (see chart D in fig. 3), (2) increasing instability of the enzyme as the pH of the medium is changed from the general region of neutrality (see table 1 and also Clarkson and Maizels, '52) and (3) a tendency for the clumping, necessary for the separation of the stromata, to be non-reproducible and frequently irreversible at pH values as acid (e.g., 4.0 to 5.5) as those commonly employed for this purpose (Ponder, '42; Parpart, '42). High concentrations of NaCl also have an agglutinating effect and are to be avoided. Before the stored stromata are used they are resuspended at 35°C. in a solution containing a low con-

centration of NaCl, at which time microscopic examination should reveal little or no agglutination.

By way of contrast with the results of the experiments summarized in table 1 is the satisfactory degree of reproducibility obtained from day to day when the standardized technique for the preparation of stromata is used. The figures in table 2 are

TABLE 1

Effect on the activities, measured under standard conditions, of stromata subjected to different pH values before and during their separation from a hemolysate of human erythrocytes. The initial concentrations of ATP were approximately 0.5 mM/L, initial total labile phosphorus 210 gamma, and the number of stromata 5×10^9.

INITIAL pH OF THE HEMOLYSATE		GAMMA OF PHOSPHORUS LIBERATED IN 30 MINUTES UNDER STANDARD CONDITIONS
(1)	7.3	5.4
(2)	*	16.7
(3)	6.5	23.6
(4)	5.9	20.3
(5)	4.6	8.3
(6)	4.3	6.3

* Represents the activity of stromata recovered from the supernatant of (1) by acidifying it to pH 6.5. It should be noted that the sum of the activities of (1) and (2) is approximately the same as (3).

TABLE 2

Comparison of the capacities of different preparations of stromata to liberate orthophosphate from ATP

MONTH	YEAR	GAMMA OF P LIBERATED IN 30 MINUTES BY 5×10^9 STROMATA	INITIAL ATP CONCENTRATION
			mM/L
Nov.	51	25.2	0.46
Dec.	51	25.2	0.50
Jan.	52	25.0 *	0.47
Jan.	52	22.8	0.42
Feb.	52	19.9	0.61
Feb.	52	23.6	0.60
April	52	24.8	0.70
April	52	25.4	0.54
April	52	21.5	1.20
July	52	26.5	0.45
Nov.	52	24.6	0.80

* Represents 3/2 of observed 20 minute values.

taken without selection from a number of independent experiments performed for other reasons over a period of a year. It should be noted that in table 2 the rate of orthophosphate formation is not clearly related, within the range employed, to the exact concentration of the substrate, ATP. It will be shown later that this result is to be expected on theoretical grounds.

Special chemicals were obtained from the following sources: a highly purified preparation of the disodium salt of ATP from the Pabst Laboratories, Milwaukee; barium salts of ADP and of muscle adenylic acid from the Sigma Chemical Co., St. Louis; barium salts of glucose 6-phosphate, and fructose 6-phosphate, as well as glycyl-glycine from the Nutritional Biochemicals Corporation, Cleveland.

All orthophosphate determinations were made by the method of Fiske and Subbarow ('25). The determination of the easily hydrolyzable phosphate of ATP, ADP and inorganic pyrophosphate was done by the acid hydrolysis method of Lohmann ('28). The sodium salts of glucose phosphate and fructose phosphate, ADP and adenylic acid were prepared from their barium salts by acidifying with HCl and then adding a slight excess of Na_2SO_4.

The following procedure was employed in the enzyme assays except where otherwise stated. In each of a series of glass centrifuge tubes are placed 5 ml of 0.045 M glycyl-glycine in 0.3% NaCl adjusted to pH 7.4, 0.1 ml of 0.5 M $MgCl_2$ and 1 ml of stroma suspension containing approximately 5×10^9 stromata. A control tube which receives 1 ml of water instead of the stroma suspension corrects for the spontaneous breakdown of ATP.

The tubes are allowed to equilibrate for 10 minutes in a water bath at 35°C. where their contents are stirred by individual glass rods operated by an electric motor. One milliliter of neutralized substrate solution is then added successively at 1.5 minute intervals to all but one of the centrifuge tubes. The last tube receives distilled water and serves as a second control to correct for any orthophosphate liberated from sources other than ATP. The total volume in each tube is now 7.1 ml. The

1.5 minute intervals provide sufficient time for removal from the tubes, after allowing approximately 20 seconds for mixing, of 3.5 ml samples representing conditions at the beginning of the incubation period. Of the sample so obtained 3.0 ml are deproteinized immediately in 3.0 ml of 16% trichloracetic acid (TCA); the remaining 0.5 ml serves for a pH determination. The TCA mixtures are quickly filtered, frozen and preserved in that state for later use (Krishnan and Nelson, '48). Finally at

TABLE 3

Changes produced by intact washed erythrocytes in the orthophosphate fraction and the acid labile phosphate fraction of an external medium having an initial composition of 0.55 mM/L of ATP in experiments 1 and 2 and 0.50 mM/L in experiment 3. The values for total labile phosphorus were 220 gamma in experiments 1 and 2 and 210 gamma in experiment 3.

	TOTAL PHOSPHORUS AS ORTHOPHOSPHATE IN GAMMA			TOTAL ACID LABILE PHOSPHORUS IN GAMMA		
	Initial	50 mins.	Increase	Initial	50 mins.	Decrease
Exp. 1	24.6	51.0	26.4	259	233	26.0
	24.6	51.0	26.4	261	233	28.0
Exp. 2	24.6	49.0	24.4	261	238	23.0
	24.6	50.0	25.4	259	233	26.0
Exp. 3	11.0	25.2	14.2	258	244	14.0
	11.0	24.8	13.8	258	246	12.0
	11.0	24.8	13.8	258	249	9.0

a chosen interval a second sample is removed and treated exactly as before. The difference between the amounts of orthophosphate in the two samples minus the amounts liberated by the two controls is a measure of the amount of ATP hydrolyzed by the enzyme system. (Results are expressed in terms of phosphorus liberated by 5×10^9 cells or stromata. Each figure in the tables represents a separate assay.)

In all cases where uninjured erythrocytes are present, the enzyme reaction is stopped by cooling the tubes in crushed ice and rapidly removing the cells by centrifugation. The supernatant is then deproteinized and treated as before. Since at experimental temperatures of 35°C. exchanges of orthophosphate between cells and medium might occur (Halpern, '36;

Hevesy, '39; Gourley, '51) measurements of increases of orthophosphate are theoretically less reliable as an index of enzyme activity than those of decreases of ATP, to which erythrocytes are generally believed to be impermeable (Hevesy and Aten, '39; Guest and Rapoport, '39). There may, however, be good agreement between the two measurements, as is shown in the data from several typical experiments in table 3.

In the case of the whole homogenates which were chiefly used by Clarkson and Maizels ('52), the situation is more complicated, since a number of compounds capable of producing orthophosphate are present in unknown and variable amounts. For this reason the use of the whole hemolysate, except in the experiment hereafter noted, was purposely avoided.

EXPERIMENTS AND RESULTS

General behavior of stromata

Relative activities of stromata and intact erythrocytes. Having available a method for converting intact erythrocytes into stromata of satisfactorily constant properties, with small numerical losses, it is of interest to compare quantitatively the respective activities of the two structures in the liberation of orthophosphate from ATP. The stromata have always proved to be considerably more active than the intact cells, as is clearly shown in table 4.

A possibly related set of facts is illustrated by another sort of experiment represented in table 5. The comparison here is one of (a) the entire hemolysate of a known number of erythrocytes with no separation of the stromata and the cell contents, (b) the stromata alone from an equal portion of the same hemolysate, and (c) the cell contents after removal of the stromata. The figures from the two concordant experiments represented in table 5 show that the stromata are more active when separated from than when in contact with the cell contents. In this connection it is of interest to note that washing the stromata under conditions which do not agglutinate them (i.e., with water) not only fails to decrease their activity but tends possibly to increase it, as is also shown in table 5.

As for the cell contents, not only do they appear to lack any demonstrable ability to dephosphorylate ATP, a conclusion already reached by Clarkson and Maizels ('52), but they seem, if anything, to have an effect in the opposite direction. In this connection it may be noted that the figures in table 5 for stromata alone exceed those for the entire hemolysate by just about the amount of orthophosphate which the cell contents

TABLE 4

Comparison of the capacities of intact cells and stromata to liberate orthophosphate from ATP. The composition of the media in the experiments represented below were as follows: In experiment 1, the initial ATP concentration was 0.49 mM/L and the initial total labile phosphorus 240 gamma. The corresponding figures in experiment 2 were 0.43 mM/L and 192 gamma; in experiment 3, 0.45 mM/L and 195 gamma.

EXPERIMENT	INCUBATION TIME	DECREASE IN ACID LABILE PHOSPHORUS	
		Intact cells	Stromata
	minutes	*gamma*	*gamma*
1	30	12.4	27.2
		11.9	26.0
		9.8	27.4
		Av. 11.4	26.9
2	30	8.0	20.0
		12.0	21.0
		8.0	20.0
		Av. 9.3	20.3
3	30	15.4	23.2
			25.6
		Av.	24.4

appear to remove. By what reactions the cell contents are able to remove orthophosphate — if indeed this effect is real — cannot be stated at present, though the conversion of orthophosphate to organic phosphate is known to be an entirely normal process in the interior of the intact erythrocyte (Hevesy and Aten, '39; Guest and Rapoport, '39).

It has not been determined why the dephosphorylating powers of stromata so greatly exceed those of intact cells.

Several possibilities suggest themselves. For example, it is conceivable that hemolysis may remove from the outer surface of the cell blocking or inhibiting materials not affected by ordinary washing. A second possibility is that as a result of

TABLE 5

A comparison of the activities of the whole hemolysate, separated and unwashed stromata, stromata washed five times in distilled water, and supernatant. In experiment 1 the initial ATP concentration was 0.42 mM/L and the total labile phosphorus 342 gamma. In experiment 2 the corresponding figures were 0.38 mM/L and 310 gamma; in experiments 3 and 4 the figures were 0.45 mM/L and 197 gamma. In each experiment the number of hemolyzed cells was 5 × 10⁹.

EXP.	TIME OF INCUBATION		WHOLE HEMOLYSATE	CHANGE IN ACID LABILE PHOSPHORUS		
				Separated stromata (unwashed)	Separated stromata (washed)	Supernatant
	minutes			*gamma*	*gamma*	*gamma*
1	40		— 24.4	— 26.8		+ 2.2
			— 23.8	— 30.2		+ 2.2
		Av.	— 24.1	— 28.5		+ 2.2
2	30		— 24.0	— 23.0		+ 1.1
			— 19.6	— 25.9		+ 1.1
			— 23.2			
		Av.	— 22.3	— 24.5		+ 1.1
3	30			— 26.9	— 26.9	
				— 26.0	— 26.9	
				— 27.0	— 26.0	
		Av.		— 26.6	— 26.6	
4	40			— 27.6	— 29.4	
				— 27.2	— 29.8	
		Av.		— 27.4	— 29.6	

hemolysis there may be a rearrangement of the sites of enzyme activity in a way that facilitates the interaction of enzyme and substrate (Potter, Recknagel and Hurlbert, '51). A third possibility is that the stromata contain enzyme molecules both at their inner and outer surfaces, only a part of which are therefore accessible to the non-penetrating molecule of ATP before

hemolysis. The fact that stromata are just about twice as active as the intact cells is possibly suggestive in this connection, though the relation may be mere coincidence.

At all events it seems likely that the dephosphorylation of ATP in the external medium occurs only at the outer surface of the intact erythrocyte. Not only have other workers (Hevesy and Aten, '39; Guest and Rapoport, '39) furnished experimental evidence of the virtual impermeability of erythrocytes to organic phosphates, including ATP, but the good agreement shown in table 3 between the decrease in acid labile phosphate (ATP) and the increase in orthophosphate in the external medium strongly suggests that the interior of the cell plays no part in the reaction in question. Other evidence for the same conclusion is presented later.

Activity of a single stroma. It will be seen later that the activity of a given suspension of stromata is directly proportional to the concentration of the latter. Since the number of stromata may be estimated in the manner already discussed, it is easy to calculate the average activity of a single stroma. Choosing for this purpose the conditions represented in table 2, it appears that each stroma during the early stages of dephosphorylation is able to liberate 3.2×10^6 molecules of orthophosphate per minute.

Treating the problem as if a single enzyme were involved, it is not yet possible to calculate a turnover number, but if it be assumed that the number is approximately that reported by Nachmannsohn ('52) for ATPase, i.e., 8,000 per minute, it would follow that the number of enzyme molecules per stroma would be of the order of 200 (assuming that the two orthophosphate groups are liberated simultaneously).

Properties of the "Stroma Apyrase"

Evidence will now be given to show that the removal of orthophosphate from ATP by the stromata of human erythrocytes has most of the chief characteristics of a typical enzyme reaction, the enzyme system involved having the properties of

an apyrase which liberates two phosphate groups from each molecule of substrate.

Theoretical considerations. The kinetics of many enzyme reactions are known to be in good agreement with the predictions from the Michaelis and Menten equation (Michaelis and Menten, '13). This equation is most commonly written in the following form:

$$v = \frac{V(S)}{K_s + (S)} \tag{1}$$

where v is the variable rate of the reaction, V is the maximum rate of the reaction, (S) is the substrate concentration, and K_s is the Michaelis affinity constant. Some of the implications of equation (1) for the present work have been put to an experimental test in the following manner.

Since V in equation (1) is known to be equal to the product of a rate constant (k) and the concentration of the enzyme in its free and combined forms together (E_{total}), it is obvious that at any one substrate concentration the initial rate of the formation of orthophosphate should be proportional to the total enzyme concentration. In the present instance, therefore, a direct proportionality between the initial rate of orthophosphate production and the number of stromata per milliliter would be expected. That this relation holds is shown in chart A of figure 1.

If desired, equation (1) may be used to calculate the Michaelis affinity constant (K_s) by graphic or other means. For greater convenience Lineweaver and Burk ('34) converted equation (1) into a linear form (2 below) which is well adapted to the treatment of the data by the method of least squares.

$$\frac{(S)}{v} = \frac{(S)}{V} + \frac{Ks}{V} \tag{2}$$

Chart B in figure 1 shows the results of two independent experiments in which the substrate concentration was varied at a single enzyme concentration. The curve in this chart illustrates that a straight line relation exists between $\frac{(S)}{v}$ and (S) as predicted from equation (2). The Michaelis constant as determined by the method of least squares from the data used

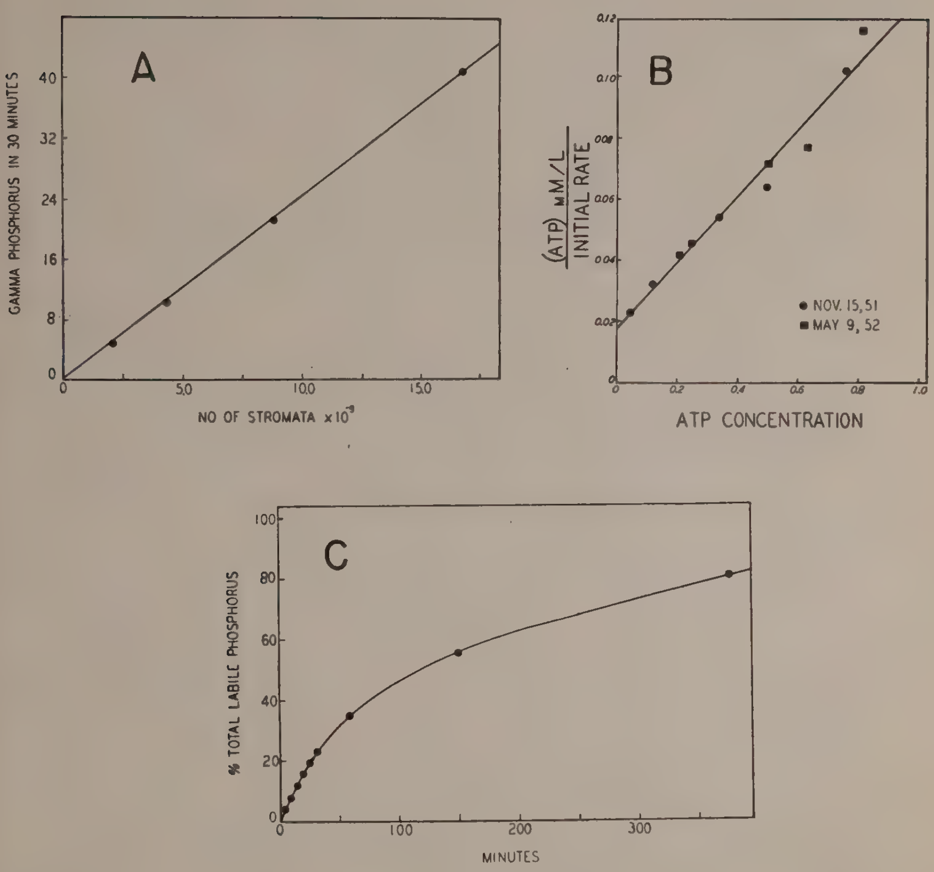

Figure 1 (A, B and C)

A. Effect of enzyme concentration (billions of stromata in 7.1 ml of suspension) on the initial rate of orthophosphate formation from ATP. Initial ATP concentration, 0.6 mM/L; initial total labile phosphorus, 226 γ.

B. Data from two experiments used to determine the Michaelis constant by the method of plotting of Lineweaver and Burk. The initial rates are measured as gamma of phosphorus liberated in 10 minutes by 5×10^9 stromata. The ATP concentration is given in mM/L.

C. Time course of liberation of orthophosphate by 5×10^9 stromata from a solution of ATP having an initial concentration of 0.38 mM/L and a total labile phosphorus content of 138 γ.

to construct chart B has a value of 2.4×10^{-4}. This may be compared with a K_s value of 1.6×10^{-4} reported by Rothstein and Meier ('48) for a similar enzyme system at the surface of the yeast cell. For comparison with other enzymes see table 6.

When the total enzyme concentration is fixed, the parameter V has a constant value. The variable observed velocity v will therefore be determined by the value of the expression $\frac{K_s + (S)}{(S)}$. Now it has already been noted in reference to table 2 that the rate of orthophosphate production within the substrate concentration range of 0.45 to 1.2 mM/L (this range for other reasons being especially convenient for the present type of experiments) is relatively constant. Calculations made by introducing the determined value of the Michaelis constant and the two values of (S) in question into the expression $\frac{(S)}{K_s + (S)}$ indicate that there should be a theoretical difference of velocity of only about 25% between these two extremes of concentration.

One of several experiments done to illustrate the time course of the enzyme reaction is represented by chart C in figure 1. Two points of interest emerge from such an experiment. The first is the linearity of the early part of the curve which is to be expected when only a small amount of the total substrate is removed during this time. The reason for choosing the half hour interval for assaying the enzyme activity is a direct result of the information obtained in experiments such as that represented in chart C. Secondly, it should be noted that after 6 hours 20% of the labile phosphorus remains undecomposed.

Specificity. The stromata used by Clarkson and Maizels ('52) were reported to decompose both ATP and diphosphoglycerate (which also contains a labile phosphate group) but neither sodium triphosphate nor sodium pyrophosphate. In the present work sodium triphosphate and diphosphoglycerate were not studied, but several other compounds were — all of a sort known to liberate orthophosphate under the influence of relatively non-specific phosphatases. Besides ATP and so-

dium pyrophosphate the list included adenosine diphosphate (ADP), fructose 6-phosphate, phosphoglyceric acid, beta-glycerophosphate, and muscle adenylic acid.

Of the substances just mentioned, when studied in the manner illustrated in chart A of figure 2, the last four were found to be affected only slightly or not at all by well-washed stromata. The behavior of ADP, on the other hand proved to be much like that of ATP. Contrary to the finding of Clarkson and Maizels ('52), the rate of decomposition of sodium pyrophosphate, though slower than that of the two adenosine polyphosphates, was by no means insignificant, as may be seen in chart A (fig. 2).

By way of possible explanation of this discrepancy, it may be noted that figures in table 1, page 116 of the paper cited show a significant rate of pyrophosphate breakdown. This activity is explained by the authors as being due to traces of pyrophosphatase carried over from the hemolysate, the enzyme in question being known to be abundant somewhere in the erythrocyte (Naganna and Narayana Menon, '48). However, stromata prepared by the present method, which involves a more thorough washing, show an even higher degree of activity. The disagreement may be due in part to the fact that Clarkson and Maizels employed with their stromata a substrate concentration which was considerably below the optimum one for erythrocyte pyrophosphatase. It is also possible that in the preparation of their stromata by the carbon dioxide method (Ponder, '42) Clarkson and Maizels ('52) may have produced considerable inactivation of the pyrophosphatase, which appears from their own work to be unusually sensitive to acidity.

The question arises whether or not the same enzyme is involved in the decomposition of ATP, ADP, and sodium pyrophosphate. An attempt was made to answer this question in the following manner. First the behavior of a given stroma suspension was studied by the method illustrated in chart B of figure 2 (1) with ATP alone, (2) with another substance — here sodium pyrophosphate — alone, and (3) the two together. The total orthophosphate liberation in (3) was then compared

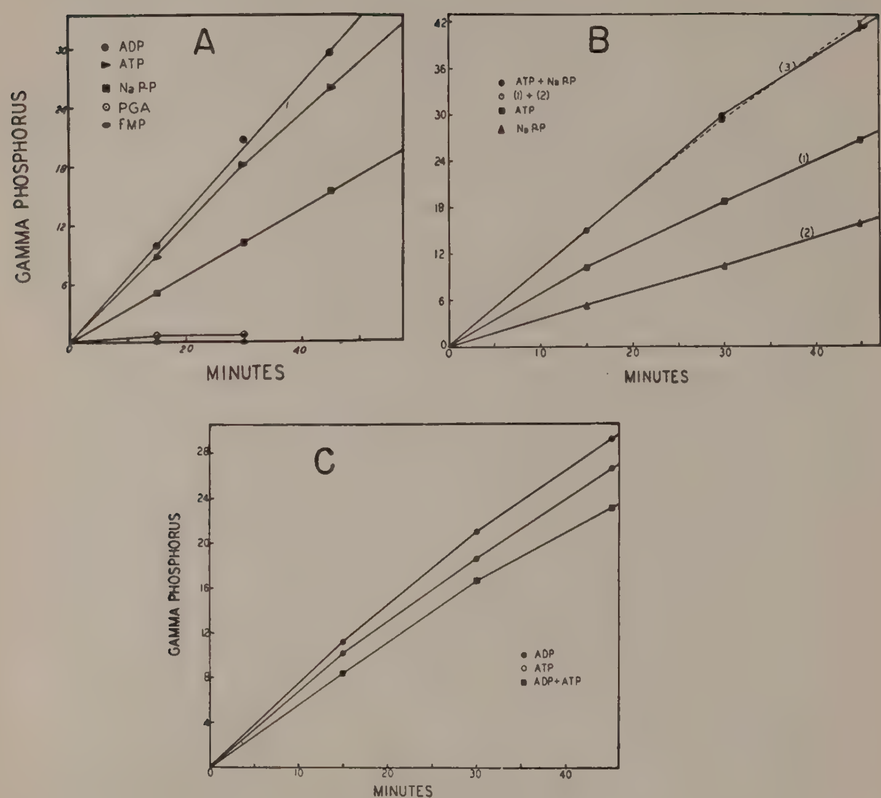

Figure 2 (A, B and C)

A. Rate of liberation of orthophosphate from solutions of 5 substrates having the following initial concentrations in mM/L: ADP and ATP, 1.2; sodium pyrophosphate, 1.2; fructose 6-phosphate (FMP), 2.0; and phosphoglyceric acid (PGA), 2.0. Curves for beta-glycerophosphate and muscle adenylic acid are not shown here since like that for FMP, they coincide with the base line.

B. Comparison of the rates of liberation of orthophosphate from ATP and sodium pyrophosphate separately (curves 1 and 2) and in combination (curve 3). The broken line represents the arithmetical sum of values from curves 1 and 2. In all cases the initial substrate concentration was 0.72 mM/L, the initial total labile phosphorus 317 γ.

C. Comparison of the rate of liberation of orthophosphate from ATP and ADP separately and in combination. For each substance the initial concentration was 0.72 mM/L. The initial total labile phosphorus for ATP was 316 γ and for ADP was 158 γ.

with the arithmetical sums of the amounts at corresponding times in curves (1) and (2). In the present instance the agreement is seen to be almost perfect. In other words, the two processes, one of which is the decomposition of ATP at its maximum rate, show complete independence of each other; they are therefore presumably governed by separate enzymes.

A similar experiment with ATP and ADP yields a result of a very different nature as may be seen in chart C of figure 2. The rates of orthophosphate liberation by the two substances under optimal and otherwise comparable conditions are much the same, but the two together give a rate so far removed from the arithmetical sum of their separate rates that it was not considered necessary to represent such a sum by a curve in chart C. These results, however, do not furnish conclusive proof that only a single enzyme is involved, since it is conceivable that, for example, the products of one reaction might interfere with the action of a second enzyme governing the other reaction.

One further question remains, namely, how the orthophosphate groups are removed from ATP. At least three possibilities suggest themselves. The first is that the two groups might be removed in succession. Another is that they could be removed together and then separated by the pyrophosphatase already mentioned, as in the case of snake venom (Zeller, 50), bone (Hitchings and Fuller, '39), and seminal fluid (Heppel and Hilmoe, '53). A third possibility is that ATP could be split to ADP, and two molecules of ADP could then be transformed into one molecule of ATP and one of adenylic acid by an enzyme similar to the myokinase described by Kalckar ('43). Entirely apart from the fact that there is still some uncertainty about the presence of a pyrophosphatase in the stromata, its slowness of action in comparison with the rate of the overall reaction (chart B, fig. 2) is hardly compatible with the idea that it forms a link in the liberation of orthophosphate from ATP (in this connection see also Gore, '51, and Rothstein and Meier, '48). If it be assumed that only one enzyme is involved in the liberation of orthophosphate from ATP, then

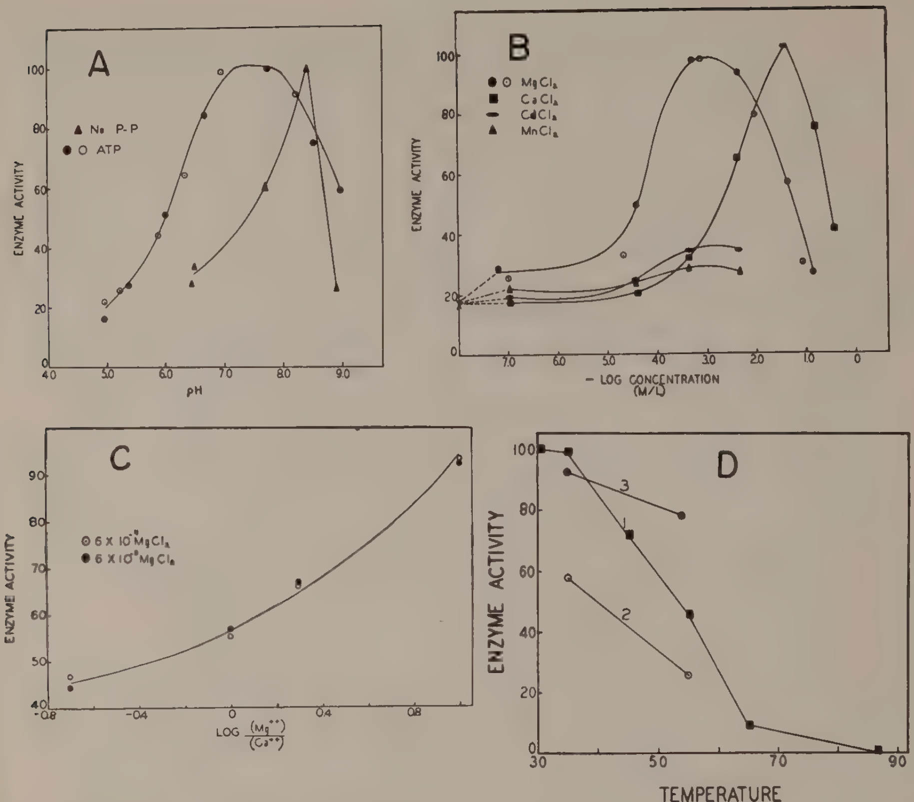

Figure 3 (A, B, C and D)

A. Effect of pH on the rate of orthophosphate formation from ATP (two independent experiments) and sodium pyrophosphate. The relative activities are expressed as percentages of a rate for ATP of 28 γ of phosphorus liberated in 40 minutes and for sodium pyrophosphate of 50 γ liberated in 30 minutes. The initial concentrations were for ATP, 0.55 mM/L; sodium pyrophosphate, 2.9 mM/L. The total labile phosphorus for ATP was 192 γ; for sodium pyrophosphate 1263 γ.

B. Effect of divalent cations on the rate of liberation of orthophosphate from ATP. All activities are expressed as percentages of an assay in which under standard conditions 16 γ of phosphorus were liberated in 30 minutes (one day old preparation of stromata was used). The initial ATP concentration in all cases was 0.80 to 0.86 mM/L, the total labile phosphorus 330 γ. The X is the activity in the absence of any added divalent cations.

C. Antagonism of the activating effect of Mg by Ca. Activities are measured as percentages of an assay of 26.5 γ of phosphorus liberated in 30 minutes under standard conditions. The initial ATP concentration was 0.60 mM/L, and the initial total labile phosphorus 273 γ.

D. Effect of preheating stromata on the rate of liberation of orthophosphate from ATP at 35°C. Curve 1, 5 minute exposure, no substrate; curve 2, 30 minute exposure, no substrate; curve 3, 30 minute exposure in the presence of ATP. All activities are expressed as percentages of an assay of 19.2 γ of phosphorus liberated in 30 minutes under standard conditions. Initial ATP concentration in all cases 0.65 mM/L, and the initial total labile phosphorus 252 γ.

the third possibility would also be eliminated; but further experimentation is necessary before definite conclusions can be drawn concerning any of these points.

pH optimum. This question has already been dealt with satisfactorily by Clarkson and Maizels ('52), who also pointed out the general similarity of the curves representing the pH dependence of the enzyme activity on the one hand and of enzyme stability on the other — a distinction that unfortunately has not always been made with enzymes in the past. As illustrated in chart A of figure 3 the results of the present work are in good agreement with those of Clarkson and Maizels ('52).

It should be noted that while the substrate studied in the experiments of Clarkson and Maizels was mixed with many other substances in a boiled hemolysate, that here employed was highly purified ATP made up in a solution of known and nearly optimum composition. As a result of experience — only a part of which is mentioned here — the pH chosen as the optimum one for most of the present experiments was 7.0 to 7.4 (for comparison with other enzymes see table 6).

Activation by Mg and Ca. It is known that these two ions have a special activating role for enzymes which dephosphorylate ATP. In some cases it is chiefly Mg which serves as the activator (Baldwin and Needham, '37; Sakov, '41; MacElroy, '47; Kielley and Meyerhof, '48; Binkley and Olson, '50), in others it is Ca (Bailey, '42; Krishnan, '49), and in still others both ions may be effective (Dubois and Potter, '43; Humphrey and Humphrey, '50).

According to Clarkson and Maizels, "stroma apyrase" would fall into the first class, since they found no activating effect of Ca. However, both Ca and Mg are here seen in chart B (fig. 3) to be effective, but over different concentration ranges. It happens that the results of Clarkson and Maizels were obtained at only one concentration of Ca (0.001 M), which lies just below the effective range shown here. It is interesting to note further that in the case of liver apyrase Dubois and Potter ('43) reported for Mg and Ca a relation which is the

TABLE 6

A comparison of the properties of the stroma enzyme system with those of similar enzymes of other tissues

SOURCE OF THE ENZYME SYSTEM	MICHAELIS CONSTANT	pH OPTIMUM	ACTIVATION		INHIBITION			NATURE OF THE ENZYME SYSTEM
			Mg	Ca	Cu	NaF	IAA *	
Water soluble extract of rabbit brain (Gore, '51)		7.4 and 8.2	++		++	+	0	possibly sulfhydryl
Water soluble extract of muscle (Kielley and Meyerhof, '48)	2.4×10^{-4}	6.5 to 7.5	++	0	++	+	+	sulfhydryl
Water soluble extract of pig brain (Binkley and Olson, '50)		7.0 to 7.5	++	0				
Muscle myosin (Bailey, '42)	5×10^{-5}	9.0 with Ca, 6.8 without Ca	—	++	+	+	0	sulfhydryl
"Stroma apyrase" (Clarkson and Maizels, '52)		7.0	++	0				
"Stroma apyrase" (present work)	2.4×10^{-4}	7.0	++	++	++	++	+	probably sulfhydryl

* Iodoacetic acid.

opposite of that observed here, i.e., maximum activation at a lower concentration for Ca than for Mg.

Three other points are brought out by chart B in figure 3. The first is that the optimum Mg concentration is approximately 7×10^{-4} to 6×10^{-3} M. The second is the virtual absence of any activating effect of Mn and Cd ions. The third is the measurable activity even in the absence of added ions, which may perhaps be attributed to traces of Mg not removed by the washing of the stromata. It should also be noted here that the presence of Na ions up to a concentration of about 0.4% has practically no effect on the activity.

It is of interest to note further that the effectiveness of Mg ions may almost completely be abolished by the addition of sufficient Ca. For example, in chart C (fig. 3), the addition of Ca ions in amounts necessary to produce the ratios plotted as abscissae reduces the effect of Mg ions until it almost disappears. Chart C also illustrates that the significant factor in this process is the ratio of the concentration of Mg ions present to the concentration of Ca ions added, since at a Mg ion concentration 10 times as great as before, the same degree of inhibition is produced by 10 times as much Ca ion. Such results suggest a case of competitive inhibition involving a single enzyme rather than two, one activated by Ca and the other by Mg, as postulated by Dubois and Potter ('43) for liver apyrase.

Inactivation by heat. The general effect of temperature on the activity of "stroma apyrase" has already been discussed (page 12); a typical experiment of more quantitative nature is presented in chart D in figure 3. In it (curve 1) samples of stromata were exposed for 5 minutes to the temperatures (°C.) indicated; their activities, expressed as percentages of that of the control, were then measured under standard conditions. The results obtained are entirely typical of enzymes in general, and in particular resemble those obtained by Jaeger and Barth ('47) with an apyrase in the frog embryo. It is known that enzymes are frequently less sensitive to high temperatures in the presence than in the absence of their sub-

strates (Spicer and Bowen, '51; Stearn, '49). That this is the case here may be seen in chart D (curves 2 and 3).

Chemical inactivation. Two kinds of inactivating agents were used in the present work. The first kind included iodoacetate, copper, and p-chloromercuribenzoate, all known to be potent inhibitors of enzyme systems that depend upon the integrity of reduced sulfhydryl groups for their action (Barron and Singer, '43, '45). These agents act either by alkylating or oxidizing the sulfhydryl group. Another kind of inactivation investigated is that produced by fluorides. These substances are believed in the presence of phosphate compounds to form complex fluorophosphates with Mg ions which play an important part in the activity of such enzymes as "stroma-apyrase."

In chart A of figure 4 are shown the results obtained with copper, iodoacetate, p-chloromercuribenzoate, and sodium fluoride. A more readily appreciated comparison than that shown in chart A may be made by noting that the concentrations required for 50% inactivation were 1×10^{-5} for copper with intact cells, 1×10^{-4} for copper with stromata, 4×10^{-5} for p-chloromercuribenzoate with stromata, 6.3×10^{-4} for sodium fluoride with stromata, and 2×10^{-1} for iodoacetate with stromata.

Several other points concerning the inactivation by copper require discussion. The first is the prevention of the copper effect by low concentrations of cysteine (not shown here). Somewhat similar results with an erythrocyte hemolysate are particularly suggestive in throwing light on the probable localization of the enzyme in the erythrocyte and on certain other peculiarities of the same cell.

For some time it has been known (Jacobs and Corson, '34) that concentrations of copper of the order of 10^{-5} M and lower strongly inhibit the uptake of glycerol by erythrocytes of man. It has also been found that this effect can readily be prevented or reversed by the hemolysate from a relatively small number of erythrocytes, whereas a larger number of intact erythrocytes have little effect. The conclusion has therefore been drawn that the copper effect is exerted at the surface of the cell

and that this substance does not enter the erythrocyte at an appreciable rate, since it otherwise would immediately be inactivated by the great excess of neutralizing substances in the interior of the cells.

The same situation exists, and the same conclusion may be drawn in the case of the inactivation of "stroma-apyrase" by

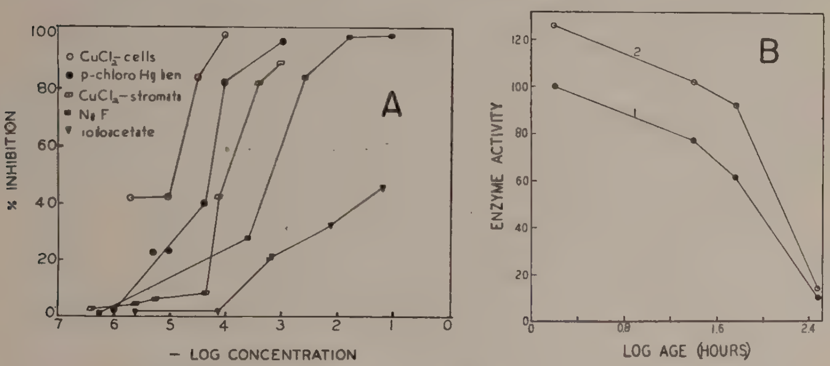

Figure 4 (A and B)

A. Effect of $CuCl_2$, NaF, p-chloromercuribenzoate, and iodoacetate on the rate of liberation of orthophosphate from ATP. The initial ATP concentration in mM/L and the initial total labile phosphorus in gamma are, respectively, for each of the above mentioned agents as follows: $CuCl_2$ (stromata), 0.40, 160; $CuCl_2$ (intact cells), 0.31, 118; NaF (stromata), 0.58, 288; iodoacetate (stromata), 0.60, 296; p-chloromercuribenzoate, 0.48, 214. The relative activities are expressed as percentages of a rate of orthophosphate formation of 18 to 24 γ of phosphorus in 30 minutes.

B. Inactivation of "stroma apyrase" by aging (curve 1, no cysteine added, curve 2, cysteine added immediately before the analysis to produce a final concentration of 0.69 mM/L). The activities are expressed as percentages of an assay of 27.0 γ of phosphorus liberated in 30 minutes under standard conditions. The initial total labile phosphorus was approximately 400 γ in each case; the initial ATP concentrations between 0.90 and 0.98 mM/L.

copper, though, it has not been possible to demonstrate any certain relation between the entrance of glycerol and the action of an enzyme on ATP. It may, however, be mentioned that the effect on both processes of pH changes in the acid direction are similar.

At all events it has been clearly shown in data not presented here that the copper effect on intact erythrocytes may be almost completely prevented or reversed by the stroma-free hemolysate from a small number of erythrocytes equal to only about 1/30 of those already present in the system. Possibly related to this result is the difference between the behavior of the stromata and of the intact cells shown in chart A (fig. 4). Presumably in the former, which are known to be far more permeable to solutes than the latter, much of the copper comes in contact with neutralizing substances that are inaccessible before hemolysis. It would seem therefore, that the present experiments not only provide additional evidence of the impermeability of the intact erythrocyte to copper, but also the localization of a large number of enzyme molecules at the outer surface of the cell — a conclusion that would already seem to be indicated by the fairly great apyrase activity of intact erythrocytes and their known virtual impermeability to ATP (Hevesy and Aten, '39; Guest and Rapoport, '39).

Effects of aging. It has already been noted by Bailey ('42), Mehl ('44), and Ziff ('44) that myosin ATPase preparations deteriorate on standing for several days, even at the pH of optimum stability of the enzyme. It has also been reported by Ziff ('42, '44) and confirmed by Mehl ('44) that the deterioration of the enzyme occurs in two stages, the first of which is reversed by cysteine or reduced glutathione.

Some of the characteristics of the aging process of "stroma-apyrase" are shown in the experiment represented in chart B of figure 4 in which a suspension of stromata was kept near the pH of optimum stability at a temperature of 4°C. for approximately 11 days. Samples were removed at 3 (control), 24, 48, and 256 hours, and the enzyme activity measured under standard conditions, both with and without the addition of cysteine.

Three main points of interest are brought out by this experiment: (a) the entirely typical deterioration of the enzyme with time; (b) the favorable effect of cysteine; and, (c) the disappearance of this effect sometime before 256 hours. These

results suggest that the deterioration involves at least two processes; one which can be seen to progress between the third and 48th hours with no significant change in the activating effect of cysteine, and the later loss of the cysteine effect itself. The first of Ziff's processes is stated to be one which is reversed by cysteine; if this process is represented here, it would seem that it must have occurred within the first three hours of the experiment.

SUMMARY

Under appropriate conditions stromata of human erythrocytes liberate orthophosphate from ATP.

1. To secure reproducible results it is necessary to avoid irreversible agglutination and other injurious effects of low pH.

2. The activity of stromata is not decreased by 5 washings in water. Stroma-free hemolysates appear to have no dephosphorylating activity.

3. The activity of the intact erythrocyte is approximately half that of its stroma. The dephosphorylation of ATP in an external solution appears to take place only at the outer surface of the cell.

4. The effects of enzyme and substrate concentration are in good agreement with the Michaelis and Menten equation.

5. The stromata also dephosphorylate ADP, and sodium pyrophosphate, but have little effect on several other related substances. ADP appears to be dephosphorylated by the same enzyme as ATP; sodium pyrophosphate by a separate enzyme.

6. Both Mg and Ca activate the enzyme, the effective concentration range for Mg being lower than that for Ca. Calcium antagonizes Mg in what appears to be a process of competitive inhibition.

7. The pH optimum is 7.0 to 7.4 and the activity is reduced in typical fashion by exposure to high temperatures.

8. Copper, p-chloromercuribenzoate, NaF, and iodoacetate inactivate the enzyme. The copper effect can be reversed by cysteine and by stroma-free hemolysates.

9. The deterioration of the enzyme with age involves at least two processes, one of which is opposed by cysteine.

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SODIUM AND POTASSIUM EXCHANGE IN CHICKEN ERYTHROCYTES [1]

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SIX FIGURES

INTRODUCTION

A basic characteristic of living cells is that they maintain an internal environment which differs from that of their surroundings. One aspect of this general problem which has been studied extensively is the maintenance of the cation imbalance between cells and their environment. Most cells have a high potassium and low sodium content when normally surrounded by a medium high in sodium and low in potassium. Many review articles have been written on this subject included: Teorell, '49; Ussing, '49, '52; Steinbach, '51 and Sheppard, '51.

The distribution of Na and K between the mammalian erythrocyte and its environment has been studied extensively. It was originally believed that the red cell membrane was impermeable to cations (see, for example, Davson and Danielli, '36), but experiments using isotopes showed that both sodium and potassium could penetrate the cell membrane (Mullins et al., '41; Harris and Maizels, '51; Sheppard et al., '51). It is now believed that a dynamic, steady state rather than an impermeable barrier accounts for the maintenance of

[1] The initial work was done with the assistance of Gladys Dart Berenda at the University of Oklahoma, where the project was supported in part by a grant RG 342C, from the U. S. Public Health Service. The more recent work was supported in part by a grant from the Faculty Research Council, Florida State University, and a grant RG 2977 from the U. S. Public Health Service. Since this manuscript was submitted for publication, a paper by M. Maizels (J. Physiol., *125:* 263, 1954) has appeared dealing with cations in chicken erythrocytes.

cation gradients across the cell surface. Such a steady state requires the expenditure of energy.

The problem of the source of this energy is still unsolved. A number of investigators have described the movements of Na and K which occur in mammalian erythrocytes incubated under a variety of conditions and several suggestions of a possible energy source have been made (Harris, '41; Maizels, '49, '51; Flynn and Maizels, '50; Danowski, '41; Weller and Taylor, '52; Soloman, '51, '52; Greig and Holland, '51; to name but a few). The last named authors in a series of papers theorized that the acetyl choline-acetyl choline esterase system is involved in supplying energy to maintain the cation distribution between erythrocytes and their environment. The other workers in general suggested that glycolysis in one way or another can supply sufficient energy to maintain the concentrations of Na and K which have been observed in mammalian erythrocytes. Since there is still no clearcut and generally accepted explanation of how these cells maintain their Na and K concentrations, a more profitable approach might be to use some other kind of erythrocyte for further study.

The non-nucleated, mammalian erythrocyte has a very low rate of respiration while the nucleated erythrocytes of lower vertebrates have a much higher respiratory rate which is more nearly comparable to that found in other tissues. If metabolic energy is involved in the distribution of cations between erythrocytes and their environment, avian erythrocytes with their higher respiratory rate might provide interesting comparative data. In addition, cation shifts in chicken erythrocytes are involved in observations previously made in our laboratory on cells incubated for many hours at 37°C. (Hunter, '49).

The present investigation describes changes in sodium and potassium content of chicken erythrocytes incubated under a variety of conditions. In all of these experiments we have observed only the movement of these ions with the concentration gradient, i.e., loss of K from the cells and gain of

Na by the cells. This is in contrast with some of the work using mammalian erythrocytes in which K moved into the cells against the concentration gradient (Harris, loq. cit.).

METHODS

Chicken blood was obtained by cardiac puncture and heparinized. In some experiments whole blood was used while in others the blood was centrifuged at approximately 2000 G, the plasma and buffy layer were removed by aspiration, and the cells washed once with Ringer Locke and resuspended in a volume of this solution equal to the volume of plasma removed. The whole blood, or cells in Ringer Locke, were then incubated; some at 37°C. or 10°C. without shaking, others at 37°C. with shaking. No glucose was added to either of the suspending media. The Ringer Locke was buffered with sodium bicarbonate while the normal blood buffers were sufficient in whole blood. A glass electrode was used to measure the pH during the course of the experiments. Bacteriologically sterile conditions were observed throughout the period of incubation.

Salt determinations. The sodium and potassium measurements were made using essentially the techniques described by Hunter ('51a).[2] An aliquot of the incubated suspension of cells in plasma or in Ringer Locke was centrifuged at approximately 2000 G. One cubic centimeter of the supernatant fluid was added to 20 cm^3 of 125 ppm Li solution. To this was added 4 cm^3 of 20% trichloroacetic acid (TCA) to precipitate the proteins. Following centrifugation, the solution was ready for analysis. One-half cubic centimeter of packed cells from the initial aliquot was added to 5 cm^3 of doubly distilled water to hemolyze the cells. Four cubic centi-

[2] There is an error in the calculations on page 704 of this paper which makes very little difference in the example given but which might make a difference in other situations. A correction was applied to the serum K reading for the amount of K contributed by the number of cells which had hemolyzed in the sample of blood. Before this correction is subtracted, the serum K value should have ben multiplied by 1 minus the hematocrit. This gets both the serum K and the correction in terms of a liter of cell suspension.

meters of TCA were then added and the solution was centrifuged. Five cubic centimeters of this supernatant fluid were then added to 20 cm³ of 125 ppm Li solution and this solution was used for analysis. In order to compensate for the fact that water as well as cations might move, all values for Na and K are calculated on the basis of the relative volume of cells and plasma (or Ringer Locke) at zero time.

Volume measurements. The hematocrit method described by Parpart and Ballentine ('43) was used to measure volume changes. It should be pointed out that as more and more

TABLE 1

Changes in chicken erythrocytes incubated at 37°C. without shaking

TIME IN HOURS	MILLIEQUIVALENT CHANGE PER LITER OF CELLS				% HEMOLYSIS		% VOLUME CHANGE	
	Na		K		P	RL	P	RL
	Plasma	Ringer Locke	Plasma	RL				
3		+ 5.3		— 0.5		2.1		+ 1.9
5		+ 10.8		— 4.0		2.2		+ 3.8
16		+ 34.6		— 33.4		8.0		+ 10.1
17	— 2.4		— 15.2				+ 5.5	
19	+ 24.5	+ 39.1	— 4.6	— 36.8	1.6	2.8	+ 13.8	+ 7.9
21		+ 43.0		— 39.0		13.2		+ 15.9
24	+ 28.0	+ 50.6	— 38.3	— 56.3	1.5	6.8	+ 7.8	+ 18.8
30	+ 133.0		— 50.1		15.1			+ 35.6
40		+ 161.0		— 70.2		53.0		+ 120.2
43½	+ 88.5		— 46.9		17.2		+ 74.2	

cells hemolyze, this method becomes more and more inaccurate. One can measure the column of cells only, omitting the ghosts, and correct this figure for the percent hemolysis. This, however, gives values which are not reliable when there is more than 10–15% hemolysis. Measuring the total length of cells plus ghosts is also unsatisfactory. Values such as the 120% increase given in the last column of table 1 are obviously too high.

Fragility measurements. The fragility of the cells was measured using a modification of the technique described by Parpart et al. ('47). Twenty cubic centimeters of the cell

suspension were added to 5 cm^3 of salt solutions which were placed in an incubator at 37°C. for an hour. Five cubic centimeters of the complement solution were then added, the suspension centrifuged, and the amount of hemoglobin in the supernatant fluid determined using a Beckman spectrophotometer. An alternative method was to add 0.1 cm^3 of the cell suspension to 5 cm^3 of the salt solutions. The rest of the procedure was the same except a Klett colorimeter with a green filter was used to measure the amount of hemoglobin.

Percent hemolysis. The amount of hemolysis was determined by centrifuging a portion of the cell suspension and withdrawing 20 mm^3 or 0.1 cm^3 of the supernatant fluid into 10 cm^3 of water. The hemoglobin was determined by using the Beckman or Klett. Considerable difficulty was encountered in obtaining a corresponding 100% hemolysis for a calibration curve. The procedure finally adopted was to add the appropriate volume of the cell suspension to 10 cm^3 of a weak barium hydroxide solution, pH 9–10.

RESULTS

Figure 1 shows the changes in cells which were incubated in Ringer Locke at 37°C. in a single container without shaking. In all of the figures the Na and K are plotted in terms of milliequivalents change. This method of plotting the data emphasizes whether or not there is an osmotically equivalent exchange of Na for K. In the first figure the salt ordinates are in terms of milliequivalents change per liter of suspension rather than per liter of cells. Since data are included for both cells and suspending fluid, these ordinates allow a direct comparison of the number of ions exchanged between the cells and their environment. These same data have been calculated in terms of milliequivalents change per liter of cells and are included in table 1. In all of the other figures the salts are plotted in terms of milliequivalents change per liter of cells.

To make it easier to compare the data, gains and losses of ions are plotted with the same sign in all of the figures. This means that in figure 1, for example, one should be able to superimpose the curve for the loss of K from the

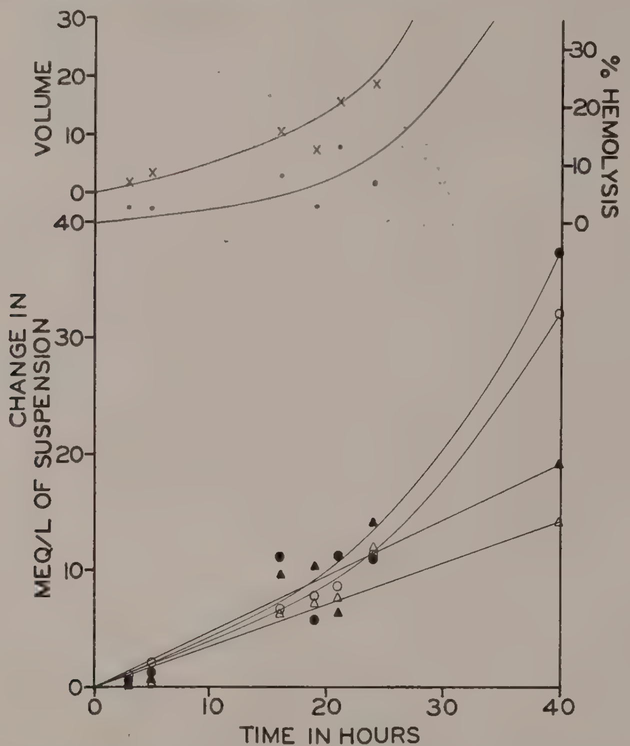

Fig. 1 Changes in chicken erythrocytes incubated in Ringer Locke at 37°C. without shaking. Values for Na and K are expressed as milliequivalents change per liter of original cell suspension. ○ — increase in Na in cells; ● — decrease in Na in suspending medium; △ — decrease in K in cells; ▲ — increase in K in suspending medium; × — percent increase in cell volumes; • — percent hemolysis.

cells upon the curve for the gain of K in the suspension medium. Similarly, the curve for Na gained by the cells should be identical with that for Na lost from the medium. If the Na and K curves are superimposable, it means there has been an osmotically equivalent exchange of one ion for

the other. Since movement of water into or out of the cells can change the concentration of ions in the cell, all values are calculated in terms of the hematocrit of the original cell suspension. In this way, the actual net number of ions which have moved can be calculated. The data obtained from several experiments have been pooled in all of the figures unless otherwise noted.

It can be seen from figure 1 that for most of the determinations there is reasonable agreement between the number of ions gained or lost by the cells and the corresponding loss or gain by the suspending medium. Also, for at least the first 24 hours, the K lost from the cells is approximately balanced by the Na gained. The values for the change in volume and percent hemolysis at 40 hours are not included in figure 1 since they are off the scale. They are given in table 1.

In table 1 the Ringer-Locke data shown in figure 1 are compared with cells standing in plasma. Both were at 37°C. Although there is considerable variability, one observes no consistent differences between cells incubated in plasma or in Ringer Locke. Up to 24 hours there is a small net loss of K from the cells and an equivalent net increase in Na. There is a slight increase in volume and a small amount of hemolysis. As the cells stand for a longer time at 37°C., the net rate of loss of K from the cells continues at approximately the same rate as during the first 24 hours. The net rate of movement of Na into the cells, however, increases at a more rapid rate than it does during the first 24 hours. After 30 or 40 hours of incubation, the cells are considerably larger and more of them hemolyze. Toward the end of these experiments when there is considerable hemolysis, it becomes difficult to determine accurately some of the values. The volume changes, for example, at 40 and 43½ hours are obviously too large.

Figure 2 shows similar data for cells incubated in Ringer Locke at 10°C. in a single, large container without shaking. Table 2 compares these data with cells incubated in plasma.

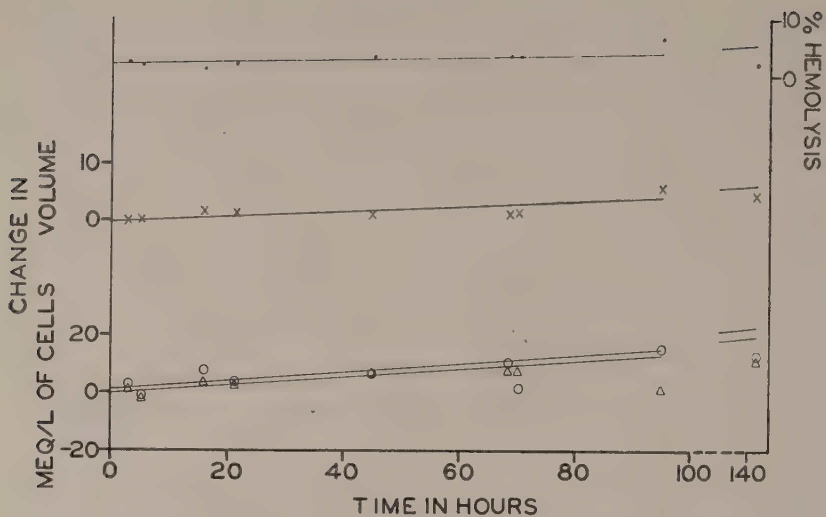

Fig. 2 Changes in chicken erythrocytes incubated in Ringer Locke at 10°C. without shaking. Values for Na and K are expressed as milliequivalents change per liter of cells which have the volume of these cells at zero time. ○ — increase in cell Na; △ — decrease in cell K; × — percent increase in cell volume; ● — percent hemolysis.

TABLE 2

Changes in chicken erythrocytes incubated at 10°C. without shaking

TIME IN HOURS	MILLIEQUIVALENT CHANGE PER LITER OF CELLS				% HEMOLYSIS		% VOLUME CHANGE	
	Na		K		P	RL	P	RL
	Plasma	Ringer Locke	Plasma	RL				
3		+ 2.4		— 2.0		2.6		0
5		— 0.7		— 0.5		2.0		0
16		+ 8.0		— 4.0		1.3		1.4
21		+ 3.7		— 3.0		2.0		1.2
22½	— 18.3		+ 19.6		—		—	
24	— 3.2		+ 6.0		—		—	
42	+ 8.1		— 17.8		—		0	
45		+ 7.5		— 7.5		3.2		1.2
54	+ 18.5		+ 8.9		—		8.5	
67	+ 32.1		+ 6.2		0	3.9	10.4	1.7
69		+ 11.4		— 9.5				
70	+ 6.7	+ 2.4	— 4.0	— 9.2	3.9	3.5	8.1	1.1
90½	+ 55.0		— 0.8		6.7		9.7	
96	+ 4.5	+ 15.4	+ 8.7	— 1.0	1.3	6.9	14.2	5.6
142		+ 13.6		— 13.0		2.4		4.7

For as long as the measurements were made, there was a small net loss of K from the cells and an equal net gain in Na. The cells changed their volume only slightly, and there was only a small amount of hemolysis. As at 37°C., there is considerable variability but no consistent differences between cells incubated in plasma and in Ringer Locke.

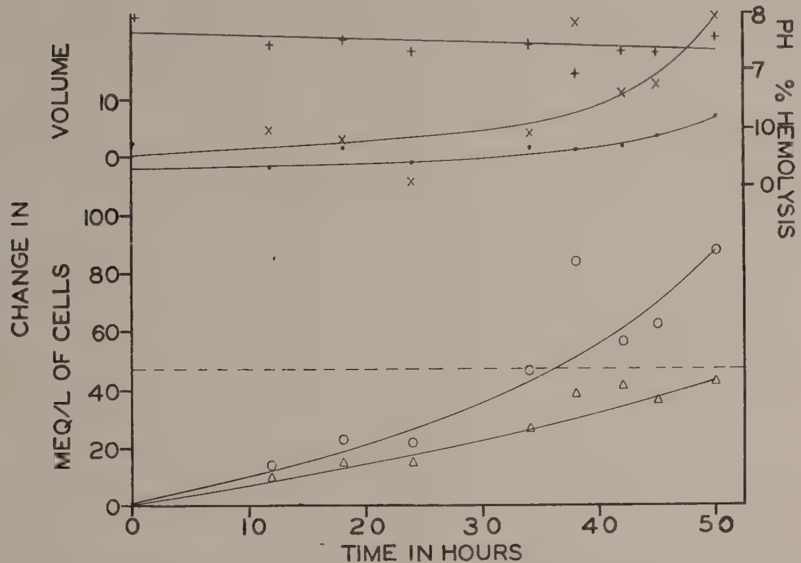

Fig. 3 Changes in chicken erythrocytes incubated in Ringer Locke at 37°C. with shaking. Values for Na and K are expressed as milliequivalents change per liter of cells of the original volume. ○ — increase in cell Na; △ — decrease in cell K; × — percent increase in cell volume; • — percent hemolysis; + — pH of cell suspension. The broken horizontal line indicates the milliequivalents of K per liter of cells which must leave the cells to give the same concentration of K in the cells and in the suspending medium.

In an additional series of experiments the effect of gentle agitation was investigated. Typical data from a single experiment in which the cells were suspended in Ringer Locke and shaken in a water bath in small containers at 37°C. are presented in figure 3. It can be seen by comparing with table 1 that gentle agitation increased the time during which the cells could be kept and also reduced the exchange of salts,

the increase in volume and the amount of hemolysis. As in the preceding experiments, there is a net loss of K from the cells at a slow, steady rate. This ion approaches diffusion equilibrium as shown in the figure by the broken horizontal line. For a time there is a net increase in cell Na which equals the loss of K. Toward the end of the experiment, the increase in cell Na is greater than the loss of cell K. There is little change in volume and only a small amount of hemolysis during the first 30–40 hours of incubation. Both of these values in-

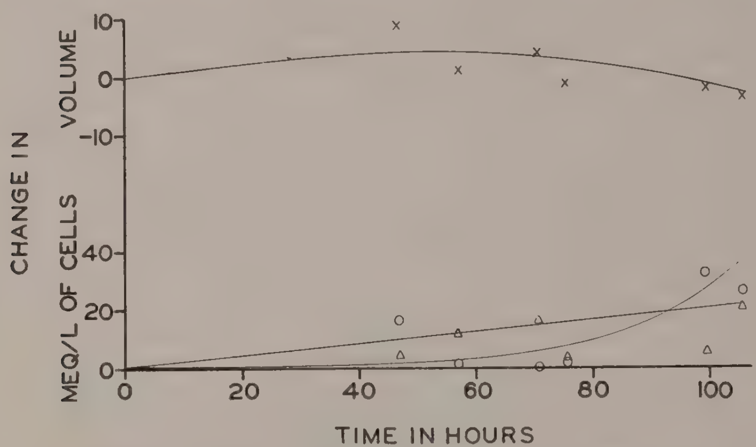

Fig. 4 Changes in chicken erythrocytes incubated in plasma at 37°C. with shaking. Values for Na and K are expressed as milliequivalents change per liter of cells of the original volume. ○ — increase in cell Na; △ — decrease in cell K; × — percent change in cell volume.

crease toward the end of the experiment as does the fragility. The pH changes only slightly during the experiments.

Before checking the effect of gentle agitation on cells suspended in plasma, the technique was changed and the procedures described by Parpart and Green ('52) for the collection of the cells for sodium and potassium analyses were followed. This method has the advantage that the cells are packed in an air turbine, and one does not have to apply a correction to the cell Na value. Data from a single experiment in which the cells were incubated at 37°C. in plasma,

with shaking are shown in figure 4. It can be seen that when the cells are shaken the changes in cells in plasma are much smaller than in cells in Ringer Locke. In plasma, the erythrocytes can be kept for over 100 hours with very little change in Na and K, in volume and only a small amount of hemolysis.

One possible explanation for the hemolysis of the cells in most of the experiments reported above might have been that eventually more Na entered than K left. This would increase the internal osmotic pressure of the cells, water would enter, the cells would swell and eventually reach their hemolytic volume. It seemed of interest, then, to incubate cells in a salt free medium. Sucrose, a non-penetrating, non-electrolyte was selected as a possible suspending medium.

After the blood had been drawn it was centrifuged, the supernatant fluid removed and the cells resuspended in a volume of 0.3 M sucrose or 0.3 M sucrose dissolved in 0.01 M NaCl or KCl. Since the results were similar in all three solutions, the data are pooled. The small amount of salt was added to reduce the stickiness of the cells which resulted from incubation in sucrose alone. Because of the difficulty in these experiments in measuring accurately quantities of cells to be analyzed, salt determinations were made on whole cells plus the sucrose solution and on the sucrose solution alone. The values for cell Na and K were obtained by subtraction. The cell suspensions were shaken during incubation.

The data obtained from several experiments are shown in figure 5. A comparison with figure 3 shows that the net loss of K from the cells in the sucrose solutions is essentially the same as it was in Ringer Locke. The horizontal line again indicates the calculated values for K in the cells at diffusion equilibrium (neglecting K in the suspending medium since this was present only in one experiment and was a very small quantity). The cell K approaches the level to be predicted at diffusion equilibrium. With little or no Na in the suspend-

ing medium the cells appear to lose this ion as well as K. As might be expected from the loss of salt, the cells usually shrink slightly during the first portion of the experiment and then swell. The amount of hemolysis as well as the fragility of the cells was variable. In general, however, there was a progressive hemolysis of the cells with time. There was no progressive change in pH during these experiments as shown by the top curve in the figure.

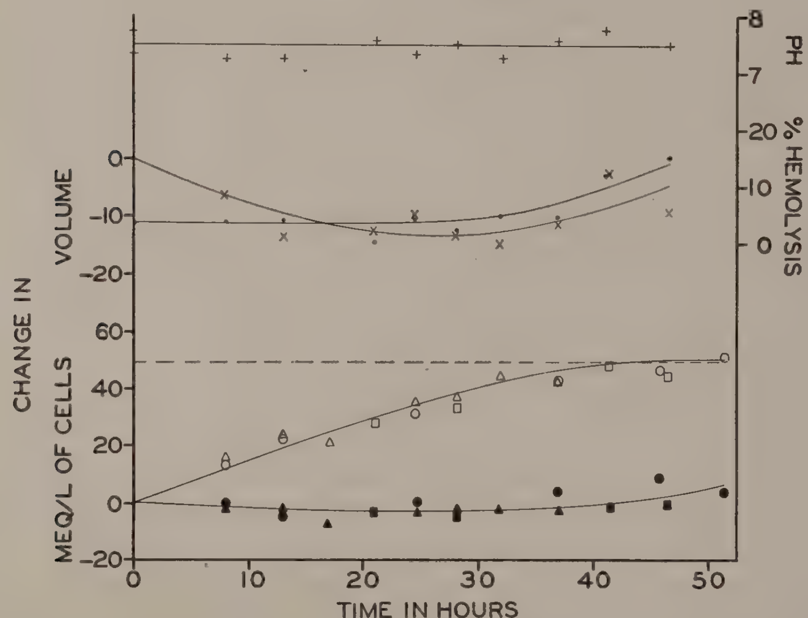

Fig. 5 Changes in chicken erythrocytes incubated in sucrose solution at 37°C. with shaking. Values for Na and K are expressed as milliequivalents change per liter of cells whose volume equals the volume at zero time. ▲ — decrease in cell Na, cells suspended in sucrose; ● — decrease in cell Na, cells suspended in sucrose plus 0.01 M NaCl; ■ — decrease in cell Na, cell suspended in sucrose plus 0.01 M KCl. △ — decrease in cell K, cells suspended in sucrose; ○ — decrease in cell K, cells suspended in sucrose plus 0.01 M NaCl; □ — decrease in cell K, cells suspended in sucrose plus 0.01 M KCl. × — percent decrease in cell volume. • — percent hemolysis. + — pH of cell suspension. The broken horizontal line indicates the milliequivalents of K per liter of cells which must leave the cells to given the same concentration of K in the cells and in the suspending medium.

DISCUSSION

These data describe in part the changes which take place under what might be called "basal" conditions of incubation of a type of nucleated erythrocyte. It must be kept in mind that movement of ions down a concentration gradient and movement of ions against the gradient may well be controlled by different mechanisms. Ion movements with the gradients, such as have been described in the present experiments, might result from: (a) some alteration in the cell membrane which would enable the ions to move more rapidly through it by diffusion; (b) a decrease in the effectiveness of the energy-yielding reactions which are responsible for moving the ions against the concentration gradient; or (c) a combination of both. The possibility exists that some information concerning the factors involved in moving ions against concentration gradients might be inferred from data such as are described in this paper.

Ponder ('49, '51) used the equation $-\frac{d\psi}{dt} = P - a\psi$ to relate these factors. This essentially says that the rate of loss of K from the cells is equal to the difference between the effect of some active process (P) which tends to accumulate K in the cell and the tendency for K to diffuse from the cell. The latter is equal to some constant, a, times the amount of K left in the cell. This equation had to be modified before the present data could be analyzed, since in some of the experiments the volume of the solution surrounding the cells was far from infinite. The second term was changed to include the concentration gradient of K rather than a function of K inside the cells.

When the data presented in figures 2 and 4 were analyzed in this way, results similar to Ponder's were obtained. The cell K appeared to be approaching new equilibrium levels. Analysis of the other experiments, however, gave considerably different results. For example, when the data shown in figure 5 were used, a negative value for P was obtained. Since the physical meaning of this is not clear, it would

suggest that this equation may not be appropriate in all cases.

A recent paper by Parpart and Green ('53) indicates the importance of pH changes. In rabbit erythrocytes there is a relationship between the metabolism of these cells, pH changes and the rate of loss of K. In mammalian erythrocytes the chief end product of glucose metabolism is lactic acid. Thus, when glucose is added to suspensions of these cells, more glycolysis occurs and the pH tends to fall. The loss of K from these cells increases as the pH decreases.

Nucleated erythrocytes in the presence of oxygen, however, have a negligible rate of glycolysis. Consequently, there is very little acid production, and under appropriate conditions of incubation the pH changes but little. The possible significance of pH changes during the incubation of chicken erythrocytes without shaking requires more detailed investigation.

One of the most obvious conclusions that can be drawn from the present data is that at lower temperatures the net change in cell Na and K occurs more slowly than at higher temperatures. One might postulate (see, for example, Harris, '41) that if metabolic energy is involved in maintaining the cation imbalance, a lowering of the temperature would decrease the rate of metabolism and so this might be expected to lead to a condition of ionic balance. The fact that this did not occur, however, does not necessarily mean that metabolic energy is not required for the maintenance of the cation imbalance.

In a previous abstract of this work (Hunter, '51b), it was suggested that hemolysis of the cells at 37°C. was a consequence of swelling due to the increase in internal osmotic pressure as more Na entered than K left.

Data obtained from several experiments are plotted in figure 6 using a different scale. This figure clearly indicates that sometime after 24 hours there is an increase in the net amount of Na which enters the cells. Potassium initially leaves the cells in a linear fashion, but then the curve levels

off as diffusion equilibrium is approached. From the steep portion of the Na curves one can estimate that approximately 1.4 m.eq. of Na are entering the cells in 1 l of blood per hour. Assuming a hematocrit of 30% this value becomes 4.7 mM of Na per hour per liter of cells. This is the same order of

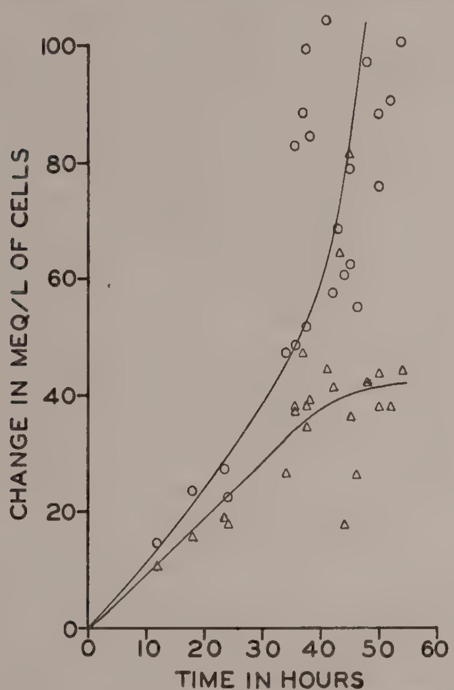

Fig. 6 Summary of changes in chicken erythrocytes incubated at 37°C. under a variety of conditions. Values for Na and K are expressed as milliequivalents change per liter of cells of volume at zero time. ○ — increase in cell Na; △ — decrease in cell K.

magnitude of movement of Na that Sheppard ('51a) reports for erythrocytes of various species of mammals.

The hemolysis which occurred in the sucrose solutions indicates that the increase in cellular cations cannot be the only mechanism involved. A few rough calculations will show that the net gain in cations observed would be expected to result in a fairly large increase in the internal osmotic pres-

sure of the cells. Assuming that a 0.3 M non-electrolyte solution is isotonic with chicken erythrocytes, a change of 30 m.eq. per liter of cells should bring about a 10% change in volume.[3] An equal exchange of Na for K would be expected to result in no net change in the number of anions in the cells. If, however, more Na enters the cell than K leaves, we might expect an equal increase in the number of anions in the cells. This means that a net increase in 15 m.eq. of cations (15.m.eq. more Na entered than K left) would be expected to result in about a 10% increase in volume. In the present experiments, the cation increases are sufficiently great to account for a considerable increase in volume.

It must also be remembered that the life span of chicken erythrocytes is only 28 days (Hevesey, '48) which means that almost 4% of the erythrocytes would be expected to hemolyze every 24 hours even "*in vivo.*"

Another possibility might be to consider the effect on cell volume of water of hydration. Assuming water moved into the cell for no other reason, the mere fact that there is considerably more water of hydration associated with a Na ion than with a K ion would make the cell swell as Na exchanges for K. A few rough calculations show that this could not contribute appreciably to the observed results (cf. Hill, '50).

The present observations, then, raise a number of interesting questions and suggest that a continuation of this study of Na and K exchange in nucleated erythrocytes may well be profitable.

SUMMARY

1. The Na and K content of chicken erythrocytes incubated at 37°C. in plasma and in Ringer Locke was determined. The cells lost K and gained Na in approximately equal amounts up to 20–30 hours. After this time they gain more Na than they

[3] If we assume that 60% of the cells is water, there are 300 m.eq. of non-electrolyte per liter of cell water or 180 m.eq. per liter of cells. A 30 m.eq. increase of non-electrolyte would require an increase of 100 ml of water per liter of cells, or a 10% increase.

lost K, they swell, they become more fragile and there is an increase in hemolysis.

2. There is very little exchange of Na and K in chicken erythrocytes in plasma or Ringer Locke kept at 10°C. for as long as 140 hours. There is very little change in cell volume and little hemolysis under these conditions.

3. If the cell suspension is gently agitated during incubation at 37°C. the changes mentioned above occur more slowly. Under these conditions cells in plasma change less than those in Ringer Locke.

4. At 37°C. the final cell K level approaches diffusion equilibrium. Cell Na, however, always is much less than the value at diffusion equilibrium.

5. Cells incubated at 37°C. in 0.3 M sucrose plus 0.01 M NaCl or KCl, lose K at essentially the same rate as cells suspended in plasma.

6. The changes described above could not be correlated with changes in pH of the suspending medium during incubation.

7. Certain theoretical implications of these data are discussed.

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SOME CHARACTERISTICS OF HEMOLYSIS BY ULTRAVIOLET LIGHT

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TEN FIGURES

The present study is concerned with the hemolysis of mammalian erythrocytes by ultraviolet light. The phenomenon may be considered as consisting of two phases. First is the direct action of the radiation on cell constituents. Second is the complex of subsequent reactions leading to the eventual release of hemoglobin. Characteristics of the first or photochemical part will be described, and evidence will then be presented to show that the characteristics of the second part are essentially consistent with the theory of colloid osmotic hemolysis (cf. Leu, Wilbrandt and Liechti, '42; Jacobs and Willis, '47; Ponder, '48; Parpart and Green, '51).

EXPERIMENTAL METHOD

Hemolysis was followed by microscopic observation and microphotography of the cells by an adaptation of the method devised by Blum and Price ('50; see method 2 of that paper) for *Arbacia* eggs. Freshly obtained human erythrocytes were used throughout. Blood was obtained by venipuncture, 2 ml being added to 0.5 ml of a buffered NaCl solution[2] in which were dissolved about 10 mg of sodium citrate. The blood cells were then washed 4 times in 10 volumes of buffered NaCl solution.

[1] Based on a thesis presented in partial fulfillment for the degree of Doctor of Philosophy in the Department of Biology, Princeton University.

[2] .139 M NaCl, .0088 M Na_2HPO_4, .0014 M NaH_2PO_4. This solution is osmotically equivalent to 0.9% NaCl and has a pH of 7.4.

The cells were held in compartments of a chamber made from three superimposed 1×3 inch microscopic slides. The bottom slide was of quartz, the middle slide of lucite, and the top slide of glass. In the lucite slide were bored three holes, each about 1 cm in diameter, which formed the compartments for holding the blood cells. Each of the slides was about 1 mm in thickness. The lucite slide was centered on the quartz slide and fastened into place by painting the edges with molten paraffin. The three compartments were then filled with the cell suspension containing approximately 3500 cells per cubic-millimeter until a large meniscus formed, and the glass slide was then placed on top. By overfilling, it was possible to avoid trapping air bubbles in the chamber. The excess fluid was then removed, and all the edges sealed with paraffin. After the chambers were filled and sealed, they were set aside for about half an hour to allow the cells to settle onto the quartz slide. In this way a single layer of cells, with no overlapping, was obtained.

After settling, the cells were irradiated from beneath with a low pressure mercury arc in a quartz envelope,[3] which lamp is credited with emitting more than 98% of its ultraviolet radiation at .2537 μ. The chamber was held at a distance of 23 cm above the lamp, and all compartments except the one being irradiated were shielded. The energy output of this lamp, as measured with a Westinghouse photocell and counting device calibrated against a U. S. Bureau of Standards lamp of known energy output, was 5.173×10^2 ergs cm^{-2} $sec.^{-1}$ for wavelengths 0.230 to 0.313 μ. When the lamp was operated with a voltage regulator, the measured intensity was found to be sufficiently constant ($\pm$ 4%) over extended periods that dosages could be measured by duration of exposure without monitoring. Evidence will be presented below to show that the wavelengths measured by the calibrating and monitoring photocells have a relatively small part in the ensuing hemolysis. For this reason, radiation doses will be listed in seconds rather than in energy units.

[3] Made by the Hanovia Chemical and Manufacturing Company, Newark, New Jersey.

Immediately after irradiation, the chamber was placed on an inverted microscope, and 4 fields in each compartment were photographed. Photographs of the same fields were taken at intervals until hemolysis was virtually complete. Subsequently, counts of the unhemolyzed cells were made from projections of the photographic negatives on a ruled screen. The initial count for each compartment was of the order of 600 to 800 cells.

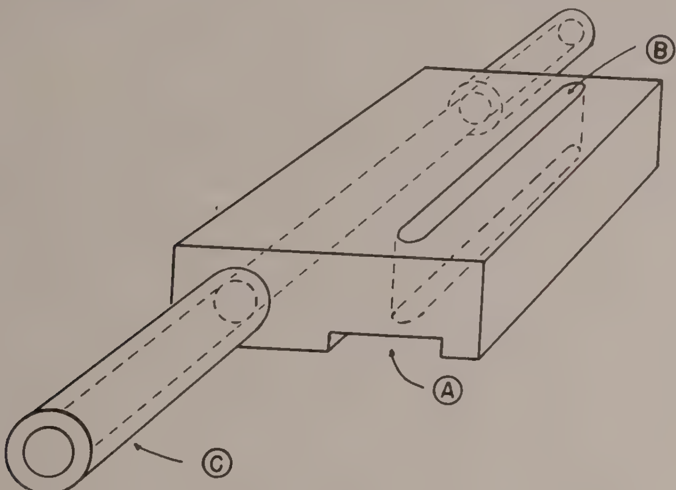

Fig. 1 Stainless steel chamber holder.

(A) Groove in which chamber is held. (B) Slit to permit illumination for photography to reach cells. (C) Leads to constant temperature water bath.

Most of the experiments were performed at room temperature (25 to 30°C.). Some experiments were carried out at 5°, 15°, and 37°, in which the temperature was controlled by mounting the chamber on the under side of a stainless steel block (fig. 1) in which a groove had been cut for holding the chamber. A slit had also been cut in the block to permit illumination for photography. Water from a constant temperature water bath was circulated through the block, about 45 minutes being allowed for temperature equilibration. To avoid condensation on humid days, a stream of N_2 that had

been passed through a coil in the water bath was directed onto the chamber during irradiation. Although between the compartments of a given chamber, dosage differences greater than 20% can be easily distinguished in terms of the resulting hemolysis curves, dose differences less than this amount cannot always be distinguished. Results obtained from different chambers cannot be directly compared, apparently because of differences in transmission of short wavelengths by the quartz slides (see below). For this reason, chambers were calibrated in terms of the effectiveness of a given dosage.

It was observed that, within a given compartment, hemolysis proceeds at a greater rate at the periphery of the compartment than at the center. Experiments in which all but a small portion of the compartments were shielded showed that this effect is independent of whether the radiation impinges on the lucite, i.e., is not due to formation of hemolysins from irradiated lucite. The enhanced hemolysis at the periphery is probably related to the optics of the system, and its effect can be largely avoided by taking measurements near the center of the compartment.

RESULTS

Shape of the hemolysis curve

The curve relating per cent of cells hemolyzed to time, is described essentially by the integral of a normal distribution curve.[4] If the time course of hemolysis is plotted on a probability grid,[5] the resultant curve is usually a straight line. Occasionally, however, the curve is convex to the abscissa, as may be seen in figure 2. Departure from linearity seems to result from a certain amount of "mechanical" hemolysis occurring during the pipetting and centrifugation of the

[4] The shape of this curve is distorted if the cells are distributed throughout the medium during irradiation instead of being in a single layer (see footnote 6, p. 72.).

[5] E.g., using either probability paper (Codex no. 3227) or converting per cent hemolysis into probability units (probits, cf. Finney, '47).

cells. This amount of hemolysis, negligible in more concentrated suspensions, occasionally goes as high as 20% in the highly dilute suspensions used in these experiments. Assuming that the cells so hemolyzed are those least resistant to osmotic hemolysis, and that ultraviolet hemolysis is

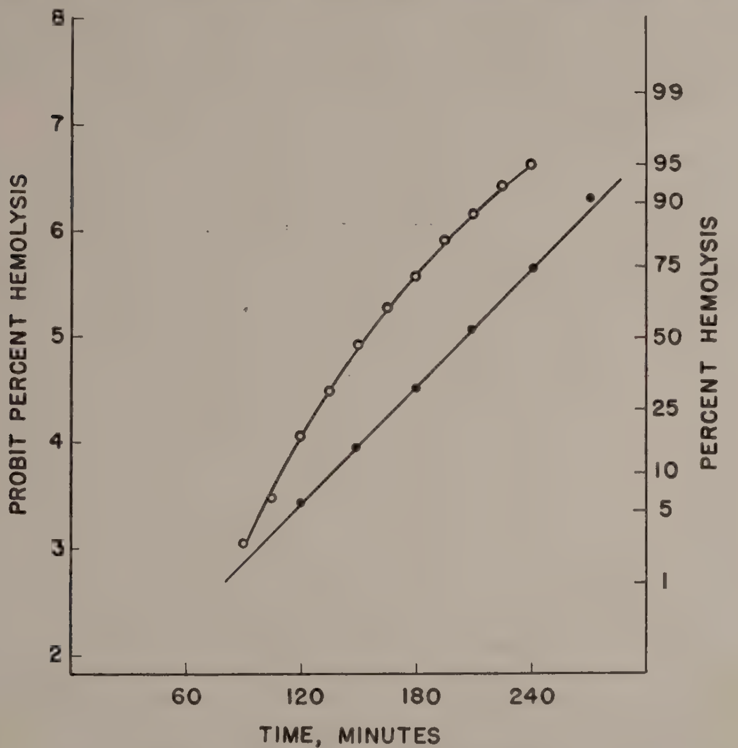

Fig. 2 Time course of hemolysis.

Two types of curves obtained when plotted on probability grid. (For discussion see text.)

a type of osmotic hemolysis (colloid osmotic hemolysis), one can straighten the convex curve in figure 2. Since the cells hemolyzed in preparation are not counted in the initial count, the "correct" initial base count can be determined by trial and error, subsequent survivor counts being unchanged. As an example, this has been done for the convex curve in

figure 2, which has been replotted in terms of the "corrected" base count in figure 3. The solid line represents actual counts, fitted by the method of least squares to probits (probably units) of the per cent hemolysis, and the dotted line the 16% least resistant cells which were lost in preparation.

For analysis, curves were plotted on a probit grid and, where necessary were "corrected." Slopes were fitted by the method of least squares to probits of the per cent hemolysis, using only the points between 15 and 85%.

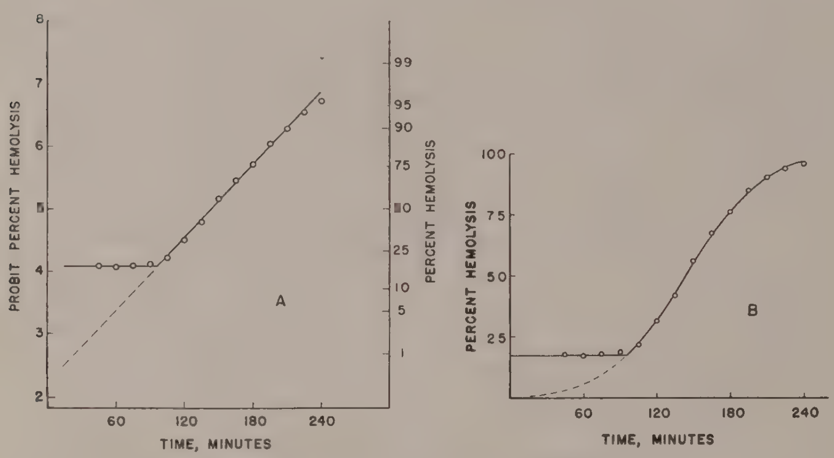

Fig. 3 Time course of hemolysis.

(A) In probability units of per cent hemolysis. Same data as used for plotting convex curve in figure 2 after "correction" for 16% initial hemolysis. Solid line fitted to actual counts; dashed line represents 16% least resistant cells lost in preparation.

(B) Same curve, replotted in terms of per cent hemolysis.

Intensity dependence

The intensity of the radiation was varied by placing blackened wire mesh screens in the light path close to the light source so as not to throw discrete shadows on the chamber containing the cells. To maintain reciprocity the time of irradiation was increased proportionally to the photometrically measured reduction in intensity by the screens, so that the dose could be held constant while the intensity was

varied. The time to 50% hemolysis was used as the criterion for the effect of a given dose.

Ordinarily, two chambers were used in each experiment. Of the three compartments in the first chamber, one was irradiated with a reference dose at the full intensity of the mercury arc (U); a second chamber was given an equivalent dose with filtered radiation (F); the third chamber was irradiated at full arc intensity, but with the dose increased by a factor of 20% over the reference dose (U + 20). The

TABLE 1

Conditions and results of reciprocity experiments

EXPT. NO.	REFERENCE DOSE U	INTENSITY OF FILTERED DOSE F	DOSE U + 20	DOSE U − 20	TIME TO 50% HEMOLYSIS MINUTES			
					U	F	U + 20	U − 20
	secs.	*rel. to U*	*secs.*	*secs.*				
E-13-I	60	.151	..	..	47	46	..	...
E-13-II	60	.151	..	..	50	75	..	...
E-14	60	.151	..	..	94	94	..	...
E-16-I	45	.151	54	..	104	81	80	...
E-16-II	45	.151	..	36	133	128	..	178
E-17-I	60	.057	72	..	101	146	59	...
E-17-II	60	.057	..	48	87	87	..	145
E-18-I	45	.057	..	36	136	124	..	164
E-18-II	45	.057	54	..	89	84	49	...
E-19-I	45	.057	..	36	115	144	..	229
E-19-II	45	.057	54	..	98	113	91	...

same procedure was followed with the second chamber except that here the third compartment received a dose 20% less than the reference dose (U-20). As mentioned previously, comparisons of raw data were made only among the three reactions in a given chamber and not between separate chambers.

In table 1, the conditions and results of the experimental series have been summarized. For the calculations, the end of irradiation was taken as the zero time. This introduced no significant error into the high intensity experiments since the time of irradiation was of the order of only one minute.

In the low intensity work, however, the time of irradiation was long (up to 18 minutes) relative to the over-all reaction time (85–150 minutes). In such cases, the zero time can only be approximated. However, since the effectiveness of the radiation is an exponential function of the radiation time at a given intensity (*cf. infra,* Dose dependence), the use of the end of the irradiation as a zero point seems a reasonable approximation. (Calculations using other zero times show

TABLE 2

Ratios of times to 50% hemolysis; reciprocity series

EXPT. NO.	$\frac{U-20}{U}$	LOG $\frac{U-20}{U}$	$\frac{F}{U}$	LOG $\frac{F}{U}$	$\frac{U+20}{U}$	LOG $\frac{U+20}{U}$
E-13-I	...	...	.98	—.009	..	...
E-13-II	...	...	1.50	.176	..	...
E-14	...	...	1.00	.000	..	...
E-16-I	...	...	.78	—.108	.77	—.114
E-16-II	1.34	.127	.96	—.018	..	...
E-17-I	...	...	1.45	.161	.58	—.237
E-17-II	1.67	.223	1.00	.000	..	...
E-18-I	1.20	.079	.91	—.041	..	...
E-18-II	...	...	.94	—.027	.55	—.260
E-19-I	1.99	.299	1.25	.097	..	...
E-19-II	...	...	1.15	.061	.93	—.031
Total logs	...	.728	...	.192	..	—.642
Av. log	...	.182	...	.017	..	—.161
Av. ratio		1.52		1.04		0.69

that this point is not a particularly significant factor in the reciprocity determinations.)

In table 2, the comparative results are presented, and the ratios of the relative times to 50% hemolysis are calculated. For the statistical analysis of these data, it was considered advisable to deal with the logarithms of the ratios rather than the actual values. By this method, the average ratio of the effects of low intensity radiation compared to those of the higher intensity radiation of equivalent dose has a

mean value of 1.04, the limits of one standard deviation being 0.85 to 1.26. Hence, the value of 1.04 agrees, within the limits of error, with the value of 1.0 expected from reciprocity. If the reference dose is compared with the dose of equal intensity but 20% less total energy, the relative time to 50% hemolysis is increased to 1.52, the limits of one standard deviation being 1.25 to 1.85. If the reference dose is compared with the dose of equal intensity but 20% greater total energy, the relative time to 50% hemolysis is decreased to 0.69, the limits of one standard deviation being 0.56 to 0.85. Use was made of the t-test to determine whether these mean ratios differed significantly. Although the number of samples was small, and the standard deviations relatively large, the F/U mean was significantly different from both the other populations at the .01 level.

It is concluded that reciprocity holds over the 18-fold range of intensities studied; that is, the rate of hemolysis is *independent of the intensity* of the incident radiation. Analysis of the slopes of the hemolysis curves leads to the same conclusion.

Dose dependence

During the early experiments it became apparent that as the dose was increased the rate of hemolysis increased more rapidly than would be expected if the rate were directly proportional to the dose. Several series of experiments were carried out to determine this relationship, first at room temperatures, then at various controlled temperatures.

In each of the room temperature series, three doses were tested, using the same chamber throughout. The dose for a given chamber was alternated so that in the three runs each dose was tested in each compartment of the chamber. Comparisons of the raw data were made only among the results of a single run. Less complete series were run at 5°, 15°, and 37°C.

As a rule the slope of the hemolysis curve was used as a measure of hemolysis. The slopes may be considered to

represent the distribution of hemolysis rates in the cell population. Rather than deal with absolute values, the slope for each of the three compartments in each chamber was determined and their ratios computed. In figure 4 the logarithm of the relative slope ratios is plotted as a function of the logarithm of the relative dose ratios. The curves are

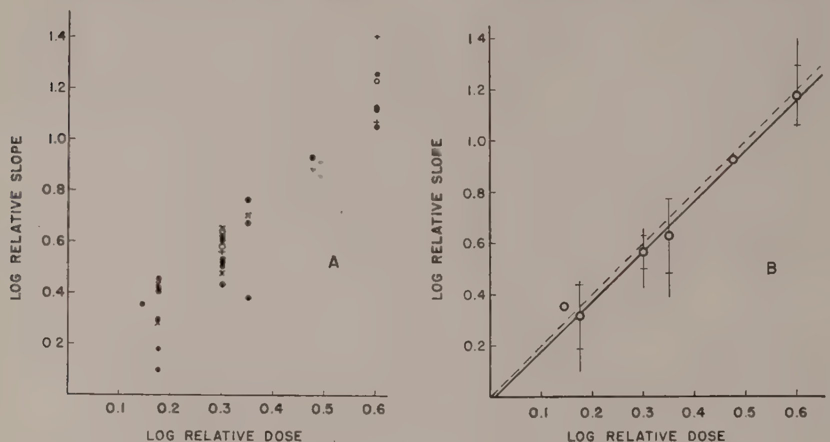

Fig. 4 Exponential relationship between dose and rate of hemolysis.

(A) Logarithm of the relative slopes of hemolysis curves as a function of the logarithm of relative doses. Data from 38 experiments at 4 temperatures:

● Room temperature (25° to 30°C.)
○ 5°C.
× 15°C.
+ 37°C.

(B) Same data as (A), showing range, mean, and standard deviation at each dose ratio.

Slope of solid curve = 1.96, calculated from the points by the method of least squares.

Slope of dashed curve = 2.0, added for comparison purposes.

based on 38 such comparisons, taken at temperatures ranging from 5 to 37°C. It is clear that temperature is without significant effect on the relationship.

At low temperatures the reaction leveled off before reaching complete hemolysis (see p. 66), and in such cases the slopes were determined from the straight portion of the curve. This was not a serious complication, since, in no

case, did the curve start to level off until hemolysis had reached at least 80%.

The solid curve in figure 4 b, determined by the method of least squares, has a slope of 1.96. The dashed curve added for comparison has a slope of 2.0. Assuming that 1.96 is not significantly different from 2.0, it may be concluded that the rate of ultraviolet hemolysis is prportional to the square of the dose. That the solid curve does not intersect the origin is interpreted as a reflection of the variability of the system, i.e., equivalent doses (ratio = 1, log ratio = 0) do not result in identical slopes.

If the time to a given per cent hemolysis is used instead of the slope as an index of the rate of hemolysis, a similar analysis may be carried out. Reciprocals of relative times to 50% hemolysis as a function of relative doses give a slope of 1.78 on a log-log plot; if 99% is used, the slope of the log-log plot is 1.90. The latter seems better justified since the times to 99% hemolysis were found by extrapolation of the points between 15 and 85%, and it is at these higher values of per cent hemolysis that the time differences between "corrected" and "uncorrected" curves are at a minimum (see figs. 2 and 3).

From these measurements it seems reasonable to represent the rate of ultraviolet hemolysis by the following equation

$$Pr(H)/t = k\,D^2$$

where Pr (H) is the probability unit corresponding to per cent hemolysis, t the time after irradiation, k a proportionality constant, and D the dose of radiation.

Zoning effect

The curve obtained by plotting time to complete hemolysis as a function of the reciprocal of concentration of lytic agent has been termed the "time-dilution" curve (Ponder, '48). Although it would be expected that the time factor would decrease as the concentration increased, a number of lysins show an irregularity in this relationship such that, as the

lysin concentration increases, the time to complete hemolysis decreases up to a certain value, then increases only to decrease again at still higher values of lysin concentration. This behavior is known as the "zoning effect." Blum, Pace and Garrett ('37) found that zoning in the time-dilution curves for the dark action of Rose bengal was markedly dependent on the temperature.

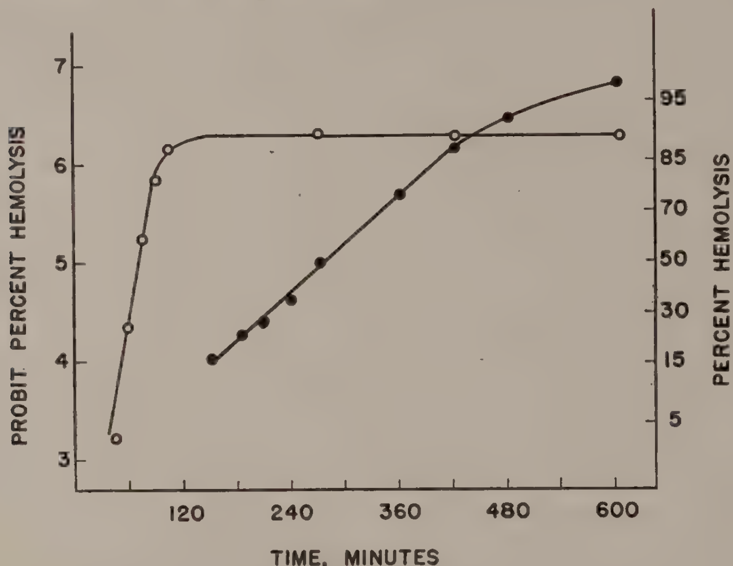

Fig. 5 "Zoning effect."

Rates of ultraviolet hemolysis at two doses at 5°C. Open circles, radiation of 120 seconds, solid circles, radiation of 60 seconds. During the straight portion of the curve, the rate at the higher dose is 4.3 times the rate at the lower dose. The curve at the higher dose, however, reaches complete hemolysis at a time considerably later than that at the lower dose.

At 5°C., indications of such a phenomenon were observed in ultraviolet hemolysis. As shown in figure 5, below 80% the relative rates of hemolysis at two doses stand in the "square" relationship to each other. However, the hemolysis curve at the higher dose breaks sharply at about 85% hemolysis and the reaction almost stops. As a result, the two hemolysis curves cross and the time to complete hemolysis is greater for the higher dose than the lower dose.

Wavelength dependence of ultraviolet hemolysis

Sonne ('27) determined an action spectrum for ultraviolet hemolysis, testing 5 wavelengths between .240 and .313 μ, and using as his criterion of effectiveness the visible diffusion of hemoglobin in blood agar during the first 20 hours after irradiation. He did not correct for the absorption characteristics of the agar. His curve indicates no maximum, but rises gradually from .313 μ to .2537 μ, and then rises sharply to .24 μ. In the present study, a complete action spectrum was not determined for ultraviolet hemolysis for technical reasons. Nevertheless, it was possible to obtain some information with respect to the wavelength dependence of the reaction.

All of the experiments discussed in this paper were carried out using the low pressure mercury arc which is credited with having a high proportion of its ultraviolet output (up to 98%) in the .2537 μ resonance line of mercury and a negligible intensity of all of the other mercury lines. The chambers were usually irradiated from below, so that the radiation impinged on the cells after traversing 1 mm of quartz and virtually no buffer solution. If the chamber were irradiated from above, so that the radiation traversed 1 mm of quartz and 1 mm of buffer solution before impinging on the cells, the dosage required for a given effect was increased about 5 times. It would seem that either of two factors might account for this difference in hemolytic effectiveness: (1) There might be a difference in the state of the cells. The cells irradiated from below are in contact with the quartz slide, and it is conceivable that at the point of contact there is a (reversible?) interaction between cell and slide which might in some way render the cell more sensitive to irradiation. (2) There might be a difference in the amount of the radiation reaching the cells. Since in both cases the radiation must pass through one quartz-air interface and one quartz buffer solution interface, the only optical difference between the systems is 1 mm of buffer solution between the cells and

light source when the radiation is from above. This layer is negligible when the cells are irradiated from below.

To test these alternatives, a comparison between "top" and "bottom" radiation was made as follows: "Top"—three quartz slides were placed over a chamber containing cells, and the whole was irradiated from above. In this case, the radiation impinged directly on the cell surface which was not in contact with the quartz. "Bottom"—a

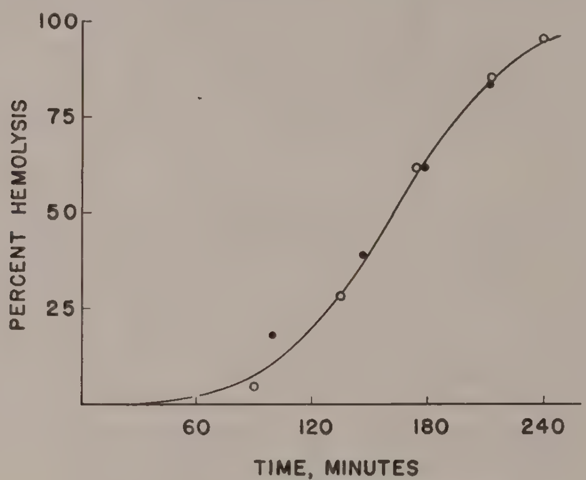

Fig. 6 "Top" vs. "Bottom" irradiation.

Curve resulting from 5 minutes irradiation through three quartz slides and 1 mm of 0.9% NaCl-PO_4.

Open circles — irradiated from above.

Solid circles — irradiated from below.

For discussion, see text.

dummy irradiating chamber was made up such that a 1 mm layer of NaCl-PO_4 was held between two quartz slides. This dummy was placed under a chamber containing cells, and the whole was irradiated from below. In this case, the radiation impinged on the cell surface in contact with the quartz. In both cases, the radiation passed through three quartz slides and 1 mm of NaCl-PO_4 before reaching the cells, and in both cases the irradiation time was 5 minutes. The results, shown in figure 6, indicate that it is immaterial whether the

cells are irradiated from above or below, and that the differences observed in the previous experiments were due to the filtering action of the 1 mm of buffer solution when the radiation was from above.

In subsequent experiments, the dummy chamber was used to hold solutions that acted as spectral filters. Three solutions were used: 0.9% NaCl-PO_4; 3% acetic acid; and distilled water. In figure 7 are plotted the spectral cut-offs of the buffer solution and the acetic acid relative to distilled

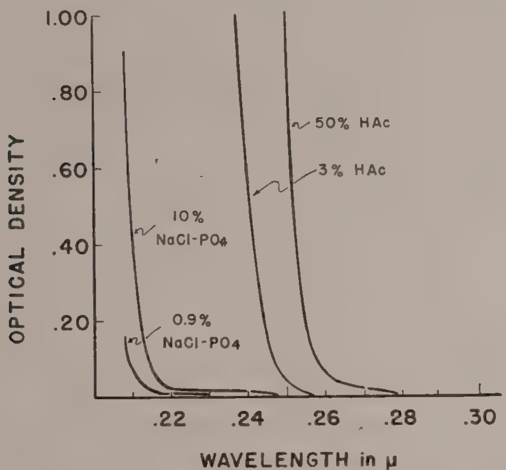

Fig. 7 Absorption of acetic acid buffered NaCl solution (NaCl-PO_4)

Absorption spectra of 10 mm thickness of solutions used as optical filters (read against distilled water standard).

water. With these filters in the light path, cells were irradiated from below for 7 minutes. The resultant hemolysis curves, shown in figure 8, indicate the effects of interposing these filters. Keeping in mind the dosages of radiation, the spectral characteristics of the substances through which the radiation passes, the relative hemolytic effectiveness of the filtered radiation, and the spectral characteristics of the low pressure mercury arc, we learn several things from these experiments. Since a 1 mm path of 3% acetic acid or 0.9% NaCl-PO_4 solution transmits more than 99% of the .2537 μ

line (and all longer wavelengths) with respect to the transmission of an equivalent path of distilled water, it is apparent from figure 8 that the .2537 μ line is not the chief factor in ultraviolet hemolysis as studied in these experiments. A consideration of the three curves in that figure in terms of those in figure 7 shows that the greater part of the effective radiation is filtered out by the acetic acid and buffer solutions. Three per cent acetic acid cuts off at about .24 μ, and 0.9% NaCl-PO_4 at about .205 μ. Both the solutions

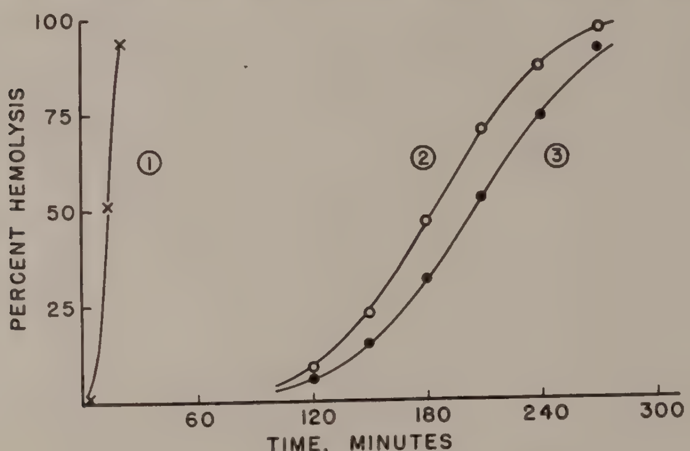

Fig. 8 Hemolysis by filtered ultraviolet radiation.

All curves resulting from 7 minutes irradiation. Filters used were 1 mm of:
(1) Distilled water.
(2) Three per cent acetic acid.
(3) 0.9% buffered NaCl solution.

seem to filter out the effective radiation to about the same degree, indicating that there is little effective radiation emitted by the low pressure mercury lamp between .205 μ and the .2537 μ line. Further, the placing of two additional fused quartz slides in the light path (as in the experiment represented in fig. 6) requires that doses be doubled in order to produce an effect equivalent to that found in experiments without the additional quartz. Allowing for 8% reflection at each surface, it may be calculated that the additional 2 mm

of quartz absorbs 30% of the effective radiation. This absorption indicates that a significant amount of the effective radiation is in the ultraviolet region of wavelengths shorter than .19 μ, where quartz begins to absorb appreciably.

Other workers using low pressure mercury arcs have found ultraviolet emission lines other than the .2537 μ line to be present, the .185 μ line (another resonance line of mercury) being especially significant. Cline and Forbes ('39) list 16 lines between .185 μ and .2537 μ, but state that only the two resonance lines are of significant intensity. Rössler and Schönherr ('38) found that the .185 μ line contributed as much as 10% of the total ultraviolet output (in energy units) of their low pressure mercury discharge tube. The contribution of the .185 μ line to the total ultraviolet emission varies considerably with the type of glass or quartz in which the mercury vapor is enclosed (cf. Koller, '52) and with the distance of the irradiated material from the source if there is an atmosphere (such as the O_2 in the air) which absorbs wavelengths of .185 μ but not .2537 μ.

Of particular interest to the present study is the work of Landen ('40) who used a low pressure lamp similar to the one employed in these experiments. Landen found that his source emitted "other mercury lines of shorter wavelength [than .2537 μ], in particular the resonance line at 1849 Å." He estimated that radiation of the latter wavelength contributed 3 to 4% to the total ultraviolet output (in quanta), which value he calculated from the chemical effects of the low pressure discharge tube radiation on urease as compared with data obtained with monochromatic radiation. He also found that the quantum efficiency for the inactivation of urease was decreased by a factor of about 4½ by the interposition of an acetic acid filter between the test solution and his low pressure arc.

In the present study, it was not feasible to measure the intensity at .185 μ. The results of the hemolysis studies, however, seem to parallel those of Landen with urease inactivation. As shown in figure 8, hemolysis following ex-

posure of cells to unfiltered radiation proceeds at a rate of about 15 times that of hemolysis following exposure of cells to radiation filtered by acetic acid or buffered solution. Calculating from the dose square factor (see p. 65), we find that the shorter line enhances the effective radiaion by a factor of about 4. Comparisons between this and Landen's factor of 4½ must be applied with caution, since the relative lengths of the air path between light source and irradiated material, which is of great importance when dealing with these short wavelengths, were probably different.

The conclusion that the short wavelengths, especially at .185μ, are highly significant in the present study of ultraviolet hemolysis seems inescapable. On the basis of equal energies, the .185μ line is apparently 100 to 200 times as effective as the .2537 μ line.

Significance attaches to these findings in that many workers have used the low pressure mercury arc with the assumption that they were dealing with a virtually monochromatic source of .2537 μ radiation. Such an assumption, which might or might not be valid, depending on the type of lamp, nature and length of the light path, etc., can easily be checked by the use of acetic acid filters. Failure to take this factor into account could lead to considerable distortion of results (see Landen, '40).

The variability of our results from slide to slide is probably related to the absorption of the .185 μ line by the fused quartz slides. Small differences in slide thickness and possible contaminants in the quartz (i.e., gold) could have a sizeable influence on the transmission of the short wavelengths, an influence which is magnified by the dose square relationships.[6]

[6] If erythrocytes are irradiated while randomly suspended in buffer solution which strongly absorbs the effective wavelengths, the resultant hemolysis curve is markedly skewed, as might be expected. Certain experiments were carried out under such conditions, and the shape of the curve analyzed (Cook, '55). The absorption by the buffer solution might well account for the shape of the hemolysis curves found by Brooks ('18).

Environmental variables and the colloid osmotic nature of ultraviolet hemolysis

Leu, Wilbrandt and Liechti ('42), studying the change in shape of the osmotic resistance curves of irradiated erythrocytes, state that ultraviolet hemolysis is colloid osmotic in nature. In the present paper, the colloid osmotic nature of short wavelength hemolysis has been re-studied, in terms of certain environmental variables.

In most of the experiments described in this section the dose was kept constant while the cell environment was varied from compartment to compartment in a single chamber. When comparing rates of hemolysis at different temperatures, this method was not feasible and the technique was modified as will be described below.

Microscopic observations. The cells were for the most part crenated at the time of irradiation, having been thoroughly washed and resuspended in an essentially protein-free medium. By the time hemolysis of irradiated cells approached 10%, the crenation had disappeared as most or all of the cells had become spheres. In a population of sphered cells, a few would be visibly larger than the rest, and these would as a rule be the first to hemolyze. The fading time of the hemolyzing cells was quite long, as much as 30 to 45 seconds in some low dose experiments. In one experiment at a fairly high dose, the fading time was of the order of 10 to 12 seconds. The over-all picture is that an irradiated cell shows no observable change for a period of time inversely related to the dose, then spheres and swells to a critical volume at which it hemolyzes, slowly fading as the hemoglobin is released. The apparent inverse relationship between dose and fading time is of interest since Ponder and Marsland (cf. Ponder, '48, p. 247) found a similar inverse relationship with respect to saponin concentration in saponin hemolysis.

Effect of osmotic pressure. Whether the critical volume of the cells was changed by the radiation was tested in-

directly by experiments in which the osmotic pressure was varied. The cells were suspended in buffered NaCl solutions of varying tonicity, allowed an hour to equilibrate with the new environment, and their hemolytic rates after a given dose of radiation then measured.

Figure 9 shows the results of an experiment in which the cells were suspended in 0.6, 0.8 and 1.0% buffered NaCl so-

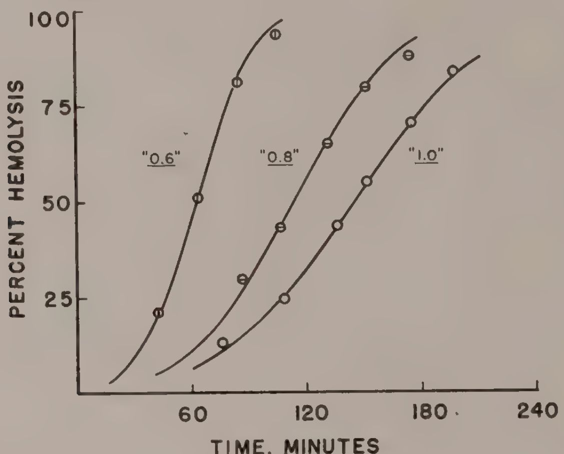

Fig. 9 Effect of external osmotic pressure on the rate of ultraviolet hemolysis.

All curves resulting from 60 seconds irradiation. Cells suspended in the following salt concentrations:

''0.6''— 0.6% NaCl-PO_4.
''0.8''— 0.8% NaCl-PO_4.
''1.0''— 1.0% NaCl-PO_4.

Curve ''1.0'' represents the best fit of the data to an integrated normal distribution curve. Curves ''0.6'' and ''0.8'' were calculated from ''1.0'' on the basis of assumptions discussed in the text.

lution. All the suspensions were irradiated simultaneously. The curve for hemolysis in 1.0% is the best fit of the data to an integrated normal distribution curve, which curve was used as the standard for calculating the other two curves. The calculations were based on a number of simplifying assumptions: (1) The critical volume for erythrocytes in hypotonic media is 170% of the initial volume (Ponder, '48,

p. 101); (2) The osmotically inactive volume of human erythrocytes is 40% (cf. Lucké and McCutcheon, '32); (3) The rate of swelling after irradiation is linear. This is an approximation, but does not seriously deviate from the published swelling curves for other types of colloid osmotic hemolysis (butyl alcohol, Parpart and Green, '51, Jacobs, '53; x-irradiation, Zumbühl, '45; photodynamic action with Rose bengal, Wilbrandt, '54). From the Boyle-van't Hoff-Mariotte relationship

$$p_1\ (v_1 - b) = p_2\ (v_2 - b)$$

where p is the external osmotic pressure, v the cell volume, and b the osmotically inactive volume, we calculate that the relative volumes of unirradiated cells in 1.0, 0.8, and 0.6% buffered NaCl are 1.0, 1.15, and 1.40 respectively. If, after irradiation, all three populations start swelling at a constant rate to a critical volume of 1.70, then the cells in the 0.8% solution should hemolyze at a rate 1.27 (0.70/0.55) times, and those in the 0.6% solution at 2.33 (0.70/0.30) times the rate of those in the 1% solution. These factors were used in drawing the curves labelled "0.8" and "0.6" from curve "1.0" in figure 9. Similar results were obtained in another experiment where the cells were suspended in 0.6 and 1.2% buffered NaCl.

The close fit between the experimental data and the calculated curves labelled "0.6" and "0.8" in figure 9 may be somewhat fortuitous, since the 4 underlying assumptions are probably not all as valid as the results might indicate. It is clear, however, that the critical volume is not significantly different from 170% of the initial volume, which is the value given for the critical volume of unirradiated cells in hypotonic solutions.

Effect of temperature. The temperature independence of the dose square relationship has already been discussed. The present section deals with the dependence of the rate of hemolysis on temperature at a given dose. For the comparison of rates of ultraviolet hemolysis at different temperatures it was necessary that different chambers be used.

The chambers were calibrated by giving identical doses to a population of cells in each chamber, the cells originating from a single sample. From the resulting curves, a correction factor was obtained. The procedure was then repeated holding one chamber at 15°C. throughout and the other at room temperature (25.0–25.7°C.). Correction for the initial decrease in cell volume at the higher temperature (Jacobs and Parpart, '31) was not made because this factor is of the order of 1 to 2% which is within the error of these calculations.

These experiments indicate a Q_{10} of approximately two for the temperature range 15 to 25°C., a value that is consistent with the semiquantitative observations made on other runs at temperatures ranging from 5° to 37°C. The conditions in these other experiments, especially the calibration of the slides and the day to day variation in cell populations, were not sufficiently controlled to give a quantitative check on the temperature coefficient. However, the temperature optimum in the region of 20°C., as reported by Leu, et al. ('42) was not observed. It is of interest that the Q_{10} found here is approximately the same as that found by Davson ('37) for the outward crossing of K^+ over the cell membrane.

Effect of hydrogen ion concentration. The pH of the suspending solutions was varied by changing the NaH_2PO_4/Na_2HPO_4 ratios. In all solutions, NaCl comprised 90% of the total salt and $-PO_4$ mixture the remaining 10%, while the tonicity maintained throughout was equivalent to 0.9% NaCl. In all pH experiments, the blood was collected and washed in buffered NaCl solution of pH 6.8. To insure constant pH, after the suspensions to be irradiated had been made up in solutions of the various pH and mounted in the chamber, the cells were allowed one hour to equilibrate. Previous experiments had shown that, in dilute suspension such as those used in this work, even after 100% hemolysis the released hemoglobin had little effect on the pH of the solution.

Figure 10 shows the results of a typical experiment. All three suspensions were exposed simultaneously to the same dose. The curves are those of the integrated normal distribution best fitted by the experimental points. In the range tested (pH 5.8–7.8) hemolysis is accelerated at the higher pH. Since an increase of one pH unit is equivalent to an increase of .02 M, or .012%, NaCl in the external environment (Jacobs and Parpart, '31), these curves should be spread further apart if they were corrected for the effect of pH on cell

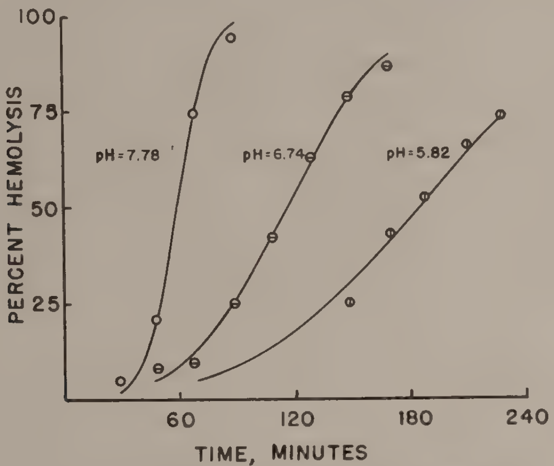

Fig. 10 Effect of pH on ultraviolet hemolysis.

All curves resulting from 70 seconds irradiation.

volume. Also, it would be expected that the rate of hemolysis would be accelerated on both sides of the isoelectric point of hemoglobin (pH 6.8), since, at the isoelectric point, the osmotic effects of the Donnan distribution are at a minimum (Netsky and Jacobs, '39), although the Donnan effect is not large in colloid osmotic hemolysis (A. K. Parpart, personal communication). Further, in the pH range 6.5 to 7.0, the K^+ permeability rate goes through a minimum (Parpart, et al., '47). As a result of these factors in a colloid osmotic system in which the initial reaction is not pH dependent, it would be expected that cells in pH 5.8 would

hemolyze much more rapidly than those at pH 7.8. In ultraviolet hemolysis, the reverse is found. This apparent discrepancy will be taken up in the discussion.

Sucrose inhibition of ultraviolet hemolysis. It is characteristic of colloid osmotic hemolysis that it can be inhibited if the external medium contains a non-penetrating non-electrolyte which offsets the internal osmotic pressure of the hemoglobin (Netsky and Jacobs, '39; Leu et al., '42; Zumbühl, '45; Jacobs and Stewart, '47; Wilbrandt, '54). Wilbrandt ('54) has developed a "compensation test" in which the cell volume does not change and hemolysis does not occur after the action of Rose bengal and light if the cells are suspended in a solution made up of .38 parts isotonic (0.3 M) sucrose and .62 parts isotonic (1.0%) NaCl. Using this "compensating concentration" an experiment was performed in which the action of the short wavelength ultraviolet hemolysis was compared for cells in the presence of sucrose and cells in isotonic buffered NaCl. The cells in buffer solution without sucrose reached 50% in 53 minutes after irradiation for 60 seconds and had passed the 90% hemolysis point, while the cells in the "compensating" sucrose solution were still crenated, i.e., had undergone little if any increase in volume and no hemolysis. Later, the cells in sucrose sphered and hemolyzed slowly, reaching 50% hemolysis in about 3½ hours.

By the criterion of the "compensation test," then, short wavelength ultraviolet hemolysis is colloid osmotic in nature. The eventual hemolysis in sucrose, however, implies more extensive damage to the cell than upsetting the selective cation permeability.

DISCUSSION

In a consideration of the photochemical reaction initiating ultraviolet hemolysis the nature of the light absorber is of first importance. Koeppe ('26) and Giaume and Paulon ('29) have claimed that the radiation is absorbed by lipids, which are converted into diffusible hemolysins. Leu et al. ('42) could

find no evidence for diffusible hemolysins, and further experiments in this laboratory have shown that unirradiated cells do not hemolyze when incubated with irradiated cells.

It is the suggestion of Leu and his co-workers that hemolysis is initiated by the denaturation of membrane proteins which have been sensitized by ultraviolet irradiation to denaturation by various anions. By analogy with work on the inactivation of enzymes with ultraviolet light, the findings presented in this paper with respect to wavelength and pH dependence are consistent with the hypothesis that the light absorbed is protein. Landen ('40) showed that the absorption coefficient of urease is 100 times greater at .185 μ than at .2537 μ, and that the quantum yield is 10 times greater at the shorter wavelength. Similar increases in quantum yield at the shorter wavelengths have also been described for the inactivation of pepsin (data of Gates, '36, recalculated by Landen, '40) and for the inactivation of trypsin (McLaren, '47). Thus, in an action spectrum for the inactivation of urease, the activity at the shorter wavelength is 1,000 times that at the longer on an incident quantum basis. This ratio is of the same order of magnitude as the relative efficiency of these two lines in ultraviolet hemolysis, where the shorter line is 100 to 200 times as effective as the longer line on an incident quantum basis.

Further evidence that the light absorber is a protein is found in the discrepancy between the pH dependence of ultraviolet hemolysis and the pH dependence of other types of colloid osmotic hemolysis. This factor suggests a marked pH dependence of the initial photochemical process which overshadows the effect of pH on the colloid osmotic mechanism. If such is the case, one would expect an amphoteric molecule to be the light absorber, i.e., a protein. Finkelstein and McLaren ('49) have shown a marked effect of hydrogen ion concentration on the quantum yield for the inactivation of chymotrypsin, as have McLaren and Pearson ('49) for the inactivation of pepsin. It seems likely that the light absorber in ultraviolet hemolysis is a protein, possibly a lipoprotein.

If we think of the red cell membrane in terms of the molecular anatomy suggested by Parpart and Ballentine ('52), we might think of the radiation induced reactions as occurring in lipoproteins lining the "pores" in the membrane, the structural characteristics of the membrane being little affected.

The hemolytic mechanism initiated by the photochemical process seems consistent with that type known as colloid osmotic hemolysis, as has been suggested by Leu et al. ('42). This mechanism may be described as consisting of essentially two phases: (1) an agent acts on the cell membrane in such a way as to alter the selective permeability to cations; (2) as K^+ and Na^+ exchange to diffusion equilibrium across the membrane, water penetrates the cell under the influence of the colloid osmotic pressure of the hemoglobin. This penetration of water results in swelling of the cell to a critical volume at which hemoglobin is released to the environment. It is the rate of K^+–Na^+ exchange which is the controlling factor in the rate of hemolysis (Parpart and Green, '51). In hemolysis by ultraviolet light, the data on Q_{10}, sucrose inhibition, and on varying the osmotic pressure of the medium all support the hypothesis that the colloid osmotic mechanism is operative here. Only the pH data seem inconsistent, which apparent inconsistency is considered to be a reflection of the effect of pH on the initial photochemical reaction, as has been discussed.

Other possible mechanisms of ultraviolet hemolysis include photolysis of the hemoglobin molecule into a number of smaller molecules with a concomitant increase in intracellular osmotic pressure, and extensive damage to the membrane leading to the loss of semi-permeability such that the membrane no longer presents a barrier to the escape of hemoglobin. It can be calculated, however, that in order to hemolyze cells in isotonic NaCl by an increase in the osmotic pressure of hemoglobin alone, it is necessary to produce 3×10^{10} osmotically active units within each red cell. Since there are 3×10^{8} molecules of hemoglobin within each cell,

and since 3×10^8 quanta of wavelength $.2537\,\mu$ impinge on each cell in 60 seconds irradiation from the low pressure mercury arc, it would be necessary that every quantum be absorbed by a hemoglobin molecule and result in the appearance of 100 osmotically active units. If only on the basis of the energy required and the energy available for such a reaction, this mechanism is clearly out of the question. The introduction of $.185\,\mu$ radiation makes these calculations only more extreme, since here a smaller number of quanta are required to produce a greater effect.

The hemolytic mechanism based on the loss of semi-permeability of the cell membrane after irradiation cannot be entirely discounted. The slow hemolysis in the presence of sucrose after irradiation indicates that the membrane has been sufficiently damaged that the normally non-penetrating sucrose molecule has penetrated the cell, initiating a delayed colloid osmotic mechanism. It is conceivable that if the colloid osmotic mechanism were completely inhibited, the radiation-induced reactions in the membrane could progress to the point where the membrane becomes premeable to the hemoglobin molecule without the intervention of osmotic factors. Such a mechanism might explain the findings of Ting and Zirkle ('40) that if swelling of erythrocytes was inhibited by sucrose after x-irradiation, slow hemolysis without volume change set in after about 36 hours. In the present work, however any such total loss of semi-permeability occurs too slowly, if at all, to be of significance.

With respect to the rate of ultraviolet hemolysis as a function of the dose of radiation, the exponent very close to two suggests that a single second order process is involved. From the present experiments it is not possible to determine whether this second order process is a characteristic of the photochemical reaction (i.e., a "two-hit" process) or of the subsequent reactions. Exponential relationship between lysin concentration and hemolysis rate in other hemolytic systems (cf. Ponder, '48; Pethica and Schulman, '53) make the latter possibility seem likely.

SUMMARY

1. A method is described for studying hemolysis after brief exposure of red blood cells to ultraviolet light.

2. After correction for the distribution of the cells' resistances to osmotic pressure, the rate of ultraviolet hemolysis appears to be a linear function of time.

3. The rate of ultraviolet hemolysis, under these conditions, is a function of the total dose of radiation and is independent of the intensity of the radiation.

4. The rate of ultraviolet hemolysis is directly proportional to the square of the dose of radiation. The exponent is independent of the temperature over the range studied, 5° to 37°C.

5. Radiation of .185 μ is of 100 to 200 times greater effectiveness in ultraviolet hemolysis than radiation of .2537 μ.

6. The Q_{10} for the rate of ultraviolet hemolysis is approximately two. Hemolysis is accelerated in solutions of decreased osmotic pressure and inhibited by the presence of sucrose in the external medium. These factors are interpreted as indicating that ultraviolet hemolysis is colloid osmotic in nature.

7. Over the pH range 5.8 to 7.8, ultraviolet hemolysis is accelerated with increasing pH. This behavior, which appears at first to be inconsistent with the theory of colloid osmotic hemolysis, is interpreted as a characteristic of the initial photochemical reaction in the cell membrane and, in this regard, is compared to certain other photochemical reactions.

8. Both the pH data and wavelength data are consistent with the hypothesis that the light absorbed is protein in nature.

ACKNOWLEDGMENTS

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BINDING OF DODECYL AMMONIUM IONS BY HUMAN ERYTHROCYTES AND ITS RELATION TO HEMOLYSIS [1]

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SEVEN FIGURES

The surface of the erythrocyte has been more thoroughly studied, by a greater variety of methods, than that of any other cell. Among the methods employed, which in part owe their existence to the ease with which the surface of this cell (ghost) can be separated from its contents, are those involving chemical analysis, optical behavior, electric impedance, electrophoresis, agglutination, hemolysis, immune reactions, antigenic properties, permeability to a variety of solutes and water, etc.

One potentially important method, so far largely neglected (see in this connection, however, Gilbert and Blum, '42 and Ponder and Ponder, '54) is the quantitative study of reversible combinations of known chemical substances, preferably of a simple nature, with the erythrocyte surface. Recent work, of which there is a considerable amount, on the attachment to the erythrocyte surface of viruses and immune bodies at present hardly belongs in this category.

[1] Part of a dissertation presented to the faculty of the Department of Physiology of the University of Pennsylvania School of Medicine in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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The present paper deals with the reversible binding by the erythrocyte surface of the cations of dodecyl ammonium chloride (DACl), a substance which is obtainable in pure crystalline form, and is quantitatively measureable by the method described below. This reagent was generously supplied by H. J. Harwood of Armour and Co., Research and Development Division, Chicago. The Orange II required for the analytic method was obtained from Prof. R. Höber and was a product of Rudolf Walter, Kiel, Germany. All the experiments were done on human blood cells, obtained as needed from a finger tip of the same individual.

Measurement of binding

Separate analyses were made of the amount of binding by the cells and of the concentration of the ligand free in the medium. The assay method [4] for DACl depends on the water insolubility of dodecyl amine (Ralston et al., '44) and the formation of an ether-soluble, water-insoluble, colored complex between the ionized form of the detergent and the anionic dye Orange II, no. 189 (Schultz, '31).

The analysis must be performed in two steps: first, extraction of the amine with ether at high pH, and second, formation of the colored complex at some pH between 3 and 8. A high pH is necessary for the initial extraction when done in the presence of cellular debris. At a pH lower than three, the dye itself becomes ether-soluble in the absence of dodecyl amine; at a pH higher than 8 or 9, the amine may not be completely converted into the dye complex; while at a pH greater than about 11 the orange dye changes to a purple form.

Scrupulous attention must be paid to the cleanliness of all glassware. Strong anionic household detergents were used to remove proteinaceous deposits, while both 95% ethanol and hot water were employed to remove detergent, as well as the

[4] This method evolved from a suggestion of Prof. I. M. Klotz, to whom I am also indebted for critical advice.

water-insoluble dye-ammonium complex. It was found to be of utmost importance that detergent be removed, otherwise satisfactory analyses for the dodecyl ammonium ion could not be performed.

Adsorption experiments were set up as follows. Suitable mixtures of a suspension of erythrocytes (either 6×10^7 or 9.4×10^7 cells per ml) and a buffered subhemolytic solution of dodecyl ammonium chloride in 0.9% NaCl were prepared in which the cell concentration and pH were held constant and only the initial DACl concentration was varied at subhemolytic levels. After equilibrium, which occurs faster than can be measured, i.e., less than 10 minutes, the cells were sedimented in a Servall Model A angle centrifuge in glass test tubes. An aliquot of the supernatant was taken for DACl analysis, some of the remaining supernatant was used for pH determination with a Cambridge Research Model pH meter, and as much of the rest of the supernatant was removed as possible without disturbing the packed cells. The latter were hemolyzed in a measured volume of distilled water. All this lysate was also analyzed for DACl.

To analyze an unknown sample it was made strongly alkaline by the addition of saturated NaOH. Dodecyl amine was exhaustively extracted with ether on a shaking machine. The ether phase was then removed and mixed with an aqueous solution of the anionic dye Orange II. The dye solution was buffered at pH 3 so that the detergent and dye formed an orange colored ether-soluble complex, the concentration of which can be measured with a Klett-Summerson photoelectric colorimeter, using the blue filter.

A blank was always run, and its value was substracted from the observed Klett readings. The corrected readings for the cell analyses were then multiplied by an appropriate factor because all the cells from a given suspension were analyzed, whereas only part of the corresponding supernatant fluid was used. For each absorption equilibrium, corrected readings for the cell and supernatant analyses were added together and

these sums plotted against the known initial DACl concentration. Figure 1 is such a graph. A straight line, fitted to the observed points by the method of least squares, permits the estimation of the unknown concentration increments, free in the medium and bound by the cells respectively.

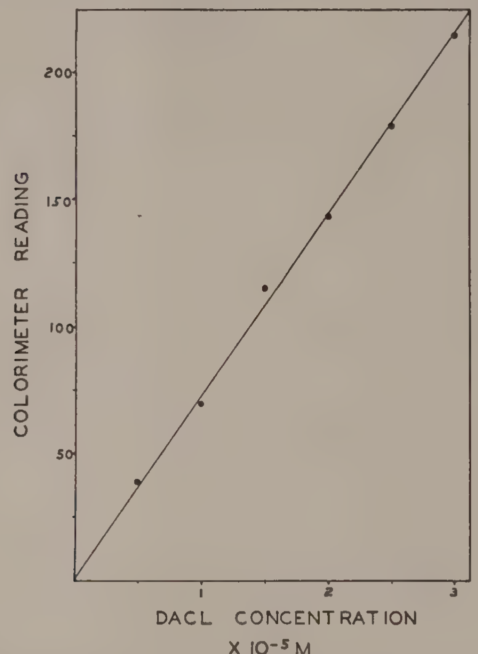

Fig. 1 The calibration curve for the analytic method for dodecyl ammonium ions, showing the relation between colorimeter reading and DACl concentration.

Reversibility of binding

That the binding process is at least to a considerable extent reversible is apparent from the ease with which dodecyl ammonium ions can be removed by washing previously equilibrated cells with a physiological salt solution. The following experiment in which the equilibrium position of adsorption and desorption are quantitatively compared shows that under the conditions there prevailing, reversibility must be virtually complete.

In this experiment suspensions of erythrocytes containing 7.5×10^7 cells per milliliter and buffered at pH 6.7 were first equilibrated with 6 different concentrations of DACl. Equilibrium concentrations of the agent in the supernatant liquid and the amounts bound per cell were determined. Two additional equilibrations at the highest concentration of DACl were performed, after which the equilibrated suspensions were centrifuged, the supernatant liquid discarded and the cells resuspended in DACl-free 0.9% NaCl of the same buffer concentration and pH as in the first equilibration. The two suspensions were then centrifuged a second time and the supernatant fluid and cells from one of them analyzed. The cells from the other suspension were then again suspended in the DACl-free buffered salt solution, the sedimentation repeated and the cells and the supernatant fluid analyzed. Obviously the last two suspensions were subjected to a single and a double desorption respectively.

The data so obtained, when plotted in figure 2 as the reciprocal of the amount bound per cell against the reciprocal of the concentration of DACl in the suspending medium, fall approximately on a straight line, with the two desorption points showing no marked departure from the general trend of the adsorption points; in fact, in view of the difficulties of the washing process, the agreement may be considered to be very good.

This method of plotting is known to yield a straight line with data described by Langmuir's adsorption isotherm (Langmuir, '18), and the line of figure 2 was obtained by the method of least squares from the data of this experiment. Isotherms similar to figure 2 have been obtained in studies of ion binding by proteins (Klotz, '49) and, when applicable, are very useful in showing at a glance the reciprocal of the theoretical maximum binding capacity by the intercept on the Y axis. The limitations of this procedure are, however, fully recognized, and it will be shown below that the linearity of the relationship is open to question.

The volume of the cellular material in this experiment was less than 1% of the volume of the suspension, and the amount of dodecyl ammonium ion taken up was between 38 and 56% of that present initially. Thus a very considerable degree of combination of the solute with the cells must have occurred.

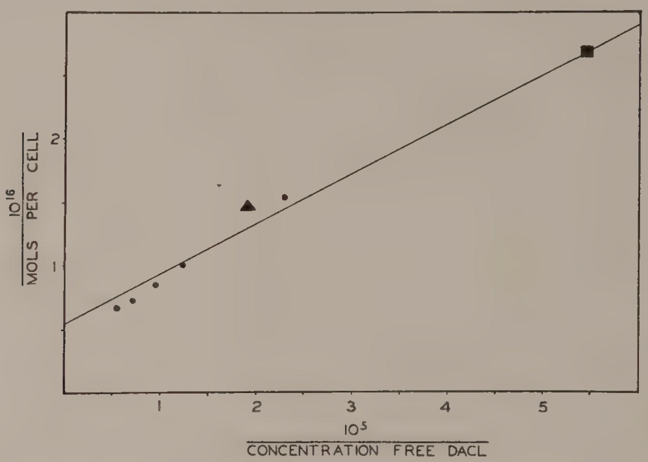

Fig. 2 Reversibility of binding. The reciprocal of the amount bound per cell plotted as a function of the reciprocal of the concentration free in the medium. Triangle obtained from the single resuspension experiment. Square obtained from the double resuspension experiment. This point coincided with one of the six original points which cannot be shown separately.

Locus of binding

That the binding of dodecyl ammonium ions by erythrocytes occurs in major part at their surfaces is shown by the respective combining powers of intact cells and their isolated surfaces, or ghosts. The latter were obtained with the angle centrifuge from a distilled water hemolysate after restoring the salinity to 0.9% NaCl. Microscopic examination showed the sedimentation to be quantitative.

Preliminary comparisons have shown that within the present limits of accuracy of the methods employed, the combining powers of intact cells and ghosts are identical at pH values from 5.5 to 8.5. As the pH increases, however,

intact cells take up more ligand from a given solution than do ghosts, until at pH 10.2 the difference amounts to about 10%. The extra combination occurring with the intact cells is accounted for by penetration of dodecyl amine into the cell and subsequent combination with intracellular hemoglobin.

The binding of dodecyl ammonium ions by hemoglobin was demonstrated as follows: Two suspensions of identical DACl, cell and buffer concentration and pH were prepared, and hemoglobin, equal in amount to that contained in the cells, was then added to one of the suspensions, the other serving as a control. Binding by the extracellular hemoglobin resulted in retention of DACl in the suspending medium. By varying the pH it was observed that as the pH was decreased, the binding of this surface-active agent by hemoglobin decreased, until at pH 8.5 it was not detectable.

That dodecyl amine may enter the erythrocyte because of its high lipid solubility is made probable by the following observations on the penetration of some dialkyl amines. Small amounts of diethyl-, dipropyl-, dibutyl-, and diamylammonium chloride were added to suspensions of washed human erythrocyte at a pH near 7. At this pH the ratio of [ion]/[base] is about 10,000/1. A few seconds later, by the addition of M/8 sodium borate the pH was changed to 9.3 and the solutions simultaneously strongly buffered at that point. This pH change greatly reduced the [ion]/[base] ratio and at the same time markedly increased the amine concentration. Following the addition of the borate the cells swelled at a rapid rate, showing that the amine form of these compounds can enter the human erythrocyte with ease (Jacobs and Parpart, '38). Presumably the same is true for dodecyl amine.

Binding capacity of the erythrocyte

The reciprocal method of plotting (fig. 2) has the advantages noted above of yielding nearly straight lines, and permitting an estimate of the binding at infinite ligand concentration by a simple extrapolation to the Y axis. On the other

hand, as units, reciprocals of concentrations and cells per mol are not immediately meaningful, though their conversion to the more obvious units of concentration and ions per cell is extremely simple. Thus in figure 2 the smallest amount of binding was 2.67×10^{16} cells per mol, with a concentration reciprocal of 5.6×10^{5} liters per mol; expressed in the more usual terms it was 2.25×10^{7} ions per cell at a concentration of 1.8×10^{-6} M. Similarly the highest binding actually observed (6.7×10^{15} cells per mol and 5.4×10^{4} liters per mol) was 8.9×10^{7} ions per cell in equilibrium with a free detergent concentration of 1.8×10^{-5} mols per liter. The maximum binding capacity determined from the y intercept in figure 2, i.e., the amount which would be taken up at infinite ligand concentration, is seen to be 5.5×10^{15} cells per mol or 1.09×10^{8} ions per cell; but for reasons given below a value of 1.3×10^{8} ions per cell appears to be preferable.

Effect of pH and temperature on binding

Adsorption isotherms (fig. 3), each obtained at a different pH but all at the same temperature, show that alkalinity favors binding, i.e., that the cell-detergent affinity increases with increasing pH. Straight lines fitted to the experimental data in figure 3 by the method of least squares give a y intercept which appears to be common to all the experiments. Thus pH does not affect the binding capacity at infinite ligand concentration. Another experiment, not plotted in figure 3, shows that the binding capacity is virtually the same at 3° and 23°C. Such observations suggest a mass action type of adsorption involving a constant maximum number of sites regardless of variations in pH and temperature, the latter parameters merely affecting the affinity of the sites for the solute.

If isotherms such as those in figures 2 and 3 can be represented by straight lines, and if they all intersect at a common point on the Y axis, interpolations on any isotherm could in theory be made if one other point on it were experimentally

known. For two reasons, however, the average value of the y intercepts in figure 3 was not used for this purpose. First, actual values of binding greater than this value have been repeatedly measured at high pH values; and second, the experimental points near the y axis lie below the fitted lines.

Therefore, ignoring the points to the right of $1/[\mathrm{DACl}] = 3 \times 10^5$, new straight lines were fitted to the data. These

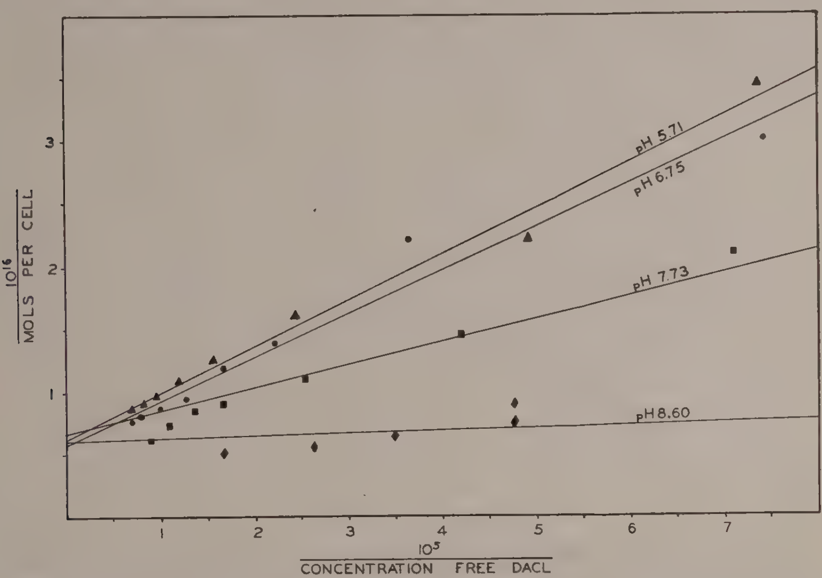

Fig. 3 The effects of pH on adsorption isotherms. Two points, one belonging to the pH 7.73 data, the other to the 8.60 data, lying off the graph to the right were also used in fitting the lines.

lines like those in figure 3 also appeared to meeet at a common y intercept; and its value of 0.45×10^{16} cells per mol (1.3×10^8 ions per cell) has been taken for interpolation in other experiments in which the concentration of free DACl is in the neighborhood of 1×10^{-5} M. Whether or not the value of the intercept adjusted in this way is a true estimate of the reciprocal of the maximum number of mols bindable per cell cannot be determined experimentally by the present

methods because, as is well known, high degrees of binding are of necessity accompanied by hemolysis.

If the only parameter varied in an adsorption experiment is pH, it is easy to show that points plotted as in figures 2 and 3 must fall on a rectangular hyperbola. This relation results from the fact that the concentration increment adsorbed plus the concentration remaining in the medium is a constant. Figure 4 is a graph of such an experiment in which only pH was varied. The points in this figure seem to lie close to the theoretical hyperbola. Since both the amount

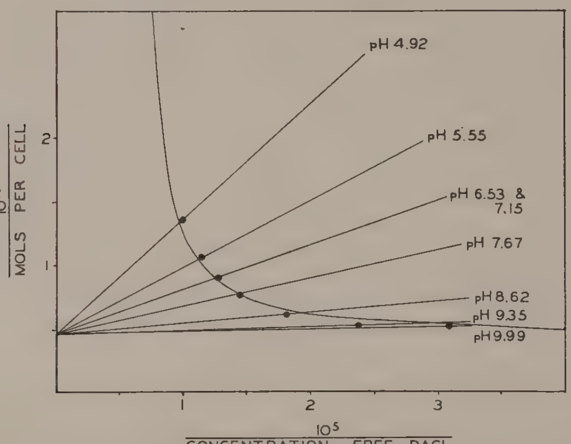

Fig. 4 The effect of variations in pH when the initial concentration of DACl is held constant.

bound per cell and the detergent concentration free in the medium were measured, it would seem from the agreement between theory and experiment that the method of analysis recovers all the ligand. It may also be concluded that no interfering substances are released from the cell surface by the binding reaction (see in this connection Ponder and Ponder, '54).

When straight lines are drawn through each of the experimentally determined points and through the adjusted average value of the y intercept of the previously mentioned isotherms, the effect of pH on binding at constant free DACl

concentration can be read directly (fig. 4). The combined effects of pH and temperature, as obtained in this manner, are shown in figure 5. The influence of temperature was determined by doing the adsorption equilibrations and subsequent centrifugations at 3°, 13°, 23° and 33°C., either in a refrigerator or an electric oven. In figure 5 the abscissae represent pH and the ordinates the fractions of the maximum binding that would have occurred at an arbitrary concentration of free DACl of 5×10^{-6} M. While figure 5 clearly shows the effect of pH on binding, it fails — probably because

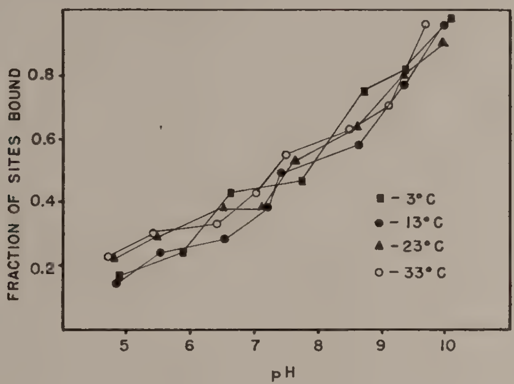

Fig. 5 The effect of pH on the fraction of sites occupied when the free DACl concentration is 5×10^{-6} M.

of lack of sensitivity of the methods used — to reveal any orderly effect of temperature.

These data indicate that temperature has a small effect on the cell-detergent affinity. In this connection it is of interest to note that according to Klotz ('49) the small influence of temperature on the ΔF of a reaction such as the ion-protein reactions studied by him indicates that the binding process is due to a favorable ΔS rather than a favorable ΔH. Since the effect of temperature on the adsorption of dodecyl ammonium ions by the erythrocyte is not large, a favorable ΔS may here also be the probable thermodynamic reason for the binding. At first sight it may seem paradoxical that an association reaction should be accompanied by a positive

entropy change; however, the paradox can be resolved by assuming with Klotz ('49) that the entropy change involved in the release of oriented water dipoles from the vicinity of both the ion and the charged site more than balances the entropy change that accompanies the actual association of the ion and the site. Since changes in the hydration of proteins are known to affect their structure (Neurath et al., '44), it can be postulated that this sort of change may perhaps underly an important previously observed effect of surface-active ions on the erythrocyte, namely the induction of a free permeability to cations (Wilbrandt, '41; Willis et al., '47; Jacobs and Stout, '50; Love, '50; and Love, '54).

The nature of the binding process

Having measured the binding of dodecyl ammonium ions as a function of the above parameters it is worth while to consider what can be inferred from these data about the binding reaction. The simplest theory compatible with these experimental results proposes that the binding occurs at two different sorts of loci on the surface of the cell, that dodecyl ammonium ions and H ions compete for the loci, and that there is no interaction between loci. One simpler theory will be shown to be inadequate; unfortunately, the information needed to test more complex hypotheses quantitatively is not available at present, but qualitatively at least the two-site theory sems adequate.

Competition between H ions and dodecyl ammonium ions for the binding sites is necessary to account for the variations of the amount bound per cell as a function of pH at constant DACl concentration. To govern the binding, reversible mass action equations of the following sort are proposed where R^- and S^- are the binding loci, which for simplicity have been assumed to be negatively charged. Thus:

$$DA^+ + R^- \rightleftharpoons DAR \qquad (1)$$
$$H^+ + R^- \rightleftharpoons HR \qquad (2)$$
$$DA^+ + S^- \rightleftharpoons DAS \qquad (3)$$
$$H^+ + S^- \rightleftharpoons HS \qquad (4)$$

Dissociation constants for (1) through (4) are respectively:

$$K_d = \frac{[DA^+][R^-]}{[DAR]}, K_h = \frac{[H^+][R^-]}{[HR]}, L_d = \frac{[DA^+][S^-]}{[DAS]},$$

and

$$L_h = \frac{[H^+][S^-]}{[HS]}$$

Concentrations appear in square brackets, and are used because activity coefficients for these quantities are unknown.

It is assumed that the total number of loci per cell is a constant, M; that a certain fraction of them, p, are of type R^-; and that the rest, $(1-p)M$, are of type S^-. Sites are accounted for as follows:

$$[R^+] + [HR] + [DAR] = \frac{npM}{N}$$

and

$$[S^-] + [HS] + [DAS] = \frac{n(1-p)M}{N}$$

where n is the concentration of cells per liter, and N is Avogadro's number. These relations can be manipulated to give:

$$\frac{n}{[DAR]} = \frac{NK_d(1 + K_h^{-1}[H^+])}{pM[DA^+]} + \frac{N}{pM} \qquad (5)$$

and

$$\frac{n}{[DAS]} = \frac{NL_d(1 + L_h^{-1}[H^+])}{(1-p)M[DA^+]} + \frac{N}{(1-p)M} \qquad (6)$$

Having obtained (5) and (6) it is appropriate to digress to show that a simpler theory with one kind of site for which H ions and dodecyl ammonium ions compete is not in accord with the data at hand. If for example there is only one sort of binding site, say R^-, then $p = 1$, and equation (5) should agree with the experimental data. At constant pH it can be rewritten with $K = K_d(1 + K_h^{-1}[H])$, and $p = 1$, thus:

$$\frac{n}{[DAR]} = \frac{KN}{M[DA^+]} + \frac{N}{M} \qquad (7)$$

This is the equation of a straight line when $n/[DAR]$ is plotted against $1/[DA^+]$. The y intercept is the reciprocal of the maximum number of mols bindable per cell. The slope is KN/M, and from the definition of K above, it should be a

linear function of the H ion concentration. But figure 6 shows it is not. These data were obtained from the average of three experiments at 23°C. of the type shown in figure 4. The non-linearity in figure 6 makes it necessary to postulate a more complex theory; one which considers binding at two sorts of loci with competition between the two kinds of ions seems to be the least complicated refinement, although over the limited concentration range here used it is not possible to state whether or not a two-site theory is adequate.

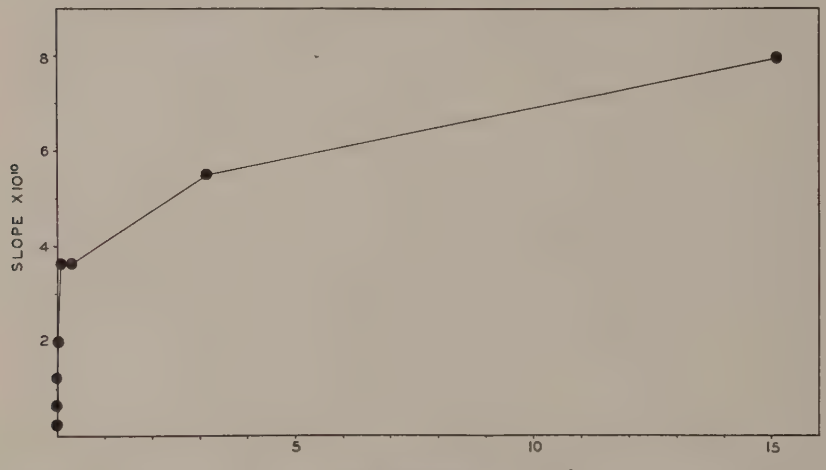

Fig. 6 The effect of H ion concentration on the slopes of some adsorption isotherms.

Experimentally the sum [DAR] + [DAS] = [DAB] is determined. At constant pH with K as above and $L = L_d (1 + L_h^{-1}[H])$; then equations (5) and (6) give:

$$\frac{n}{[DAB]} = \frac{N}{M\,[DA^+]}\left\{\frac{[DA^+]\,(Kp + L - Lp + KL)}{[DA^+] + Lp + K - Kp}\right\} + \frac{N}{M} \qquad (8)$$

In this case n/[DAB] is not linearly related to 1/[DA$^+$], but again, the y intercept is N/M. Provided the extrapolation is valid, M is thus the only one of the 6 constants in (8) which can be estimated from the present data. On the other hand, equation (8) is quite complicated enough to describe the pres-

ent results. An independent estimate of the values of K_h, L_h, p and M could theoretically be obtained by measuring the binding of H ions alone as a function of the H ion concentration, provided that all groups which bind H ions also bind dodecyl ammonium ions.

The major experimental difficulty in such a measurement is the high permeability of the erythrocyte to anions. Because binding of dodecyl ammonium ions is thought to be at the cell surface, it is likewise necessary to measure binding of H ions by the surface uncomplicated by anion exchanges with the interior of the cell. These measurements could theoretically be made by separating the two processes in time, i.e., by determining H ion binding, which presumably is an enormously fast ionic reaction, before anion exchanges have time enough to alter the H ion concentration.

A flow system similar to that used by Steinhardt and Zaiser ('51) with a time of resolution of 0.1 second was therefore constructed, in which a suspension of erythrocytes and a solution of HCl in 0.9% NaCl were rapidly run down the condenser type glass electrode of a Cambridge pH meter at constant flow velocity. Preliminary experiments with this system have shown that H ions are in fact bound appreciably at the cell surface, but the present apparatus is not of proper design to permit the accumulation of reliable quantitative data, and thus the evaluation of the constants involved in equation (8) has not yet been practicable.

The assumption that the dodecyl ammonium ion rather than dodecyl amine is the ligand has been made throughout this paper. The data presented show that at least two H ion dissociation constants are operative in the binding. If one is assigned to the ammonium-amine conversion, the other must be part of the binding reaction. Since it is difficult to see how an undissociated molecule can compete with H ions for the binding site, the binding of dodecyl amine as such is rejected unless better data should necessitate its assumption. Such considerations also suggest that cholesterol, with no dissociating groups, is an unlikely site for binding dodecyl ammonium

ions (Pethica and Schulman, '53), and therefore that components of the erythrocyte surface other than cholesterol would repay investigation by their techniques.

The relation between binding and hemolysis

It has been shown that alkalinity strongly favors the binding of dodecyl ammonium ions by the erythrocyte and it has also been reported (Love, '54) that alkalinity favors hemolysis by DACl. In view of the similarity of the effects of pH on the two processes, it may tentatively be postulated that the amount of the permeability change that is responsible for hemolysis is quantitatively related to the amount of lytic agent adsorbed.

This hypothesis has been tested as follows. The concentration of DACl was first determined which at a given pH value produced an arbitrarily selected time-course of hemolysis. By repeating the experiment at other pH values the relation between the concentration of DACl required to reproduce the arbitrary hemolysis curve and the pH of the solution was obtained (fig. 7). Very dilute cell suspensions were employed in order that binding of the hemolysin by the cells would not alter its concentration in the medium significantly. Rapid hemolysis was likewise used in the hope that complications of the antihemolytic action of DACl would be avoided, since little "protection" would be expected in the times involved, which were of the order of 15 seconds (Love, '54). Also represented in figure 7 as a function of pH is the concentration of DACl in the medium necessary for the binding of a selected amount of dodecyl ammonium ions by the cell. The values plotted are the averages of three experiments on binding at 23°C., of which figure 5 is an example. The logarithms of the DACl concentrations are used because they are more convenient.

The amount of agent bound per cell in figure 7 was so chosen that minimum deviations occurred between the binding and hemolysis curves. Under these conditions it will be

observed that the widest differences between the two curves are not greater than half a logarithmic unit, and therefore the hypothesis of equal hemolytic effects with an equal degree of binding might account for the observed results within a factor of 3 or 4 over a range of DACl concentration of 100 and of H ion concentration of 100,000. It must be noted, however,

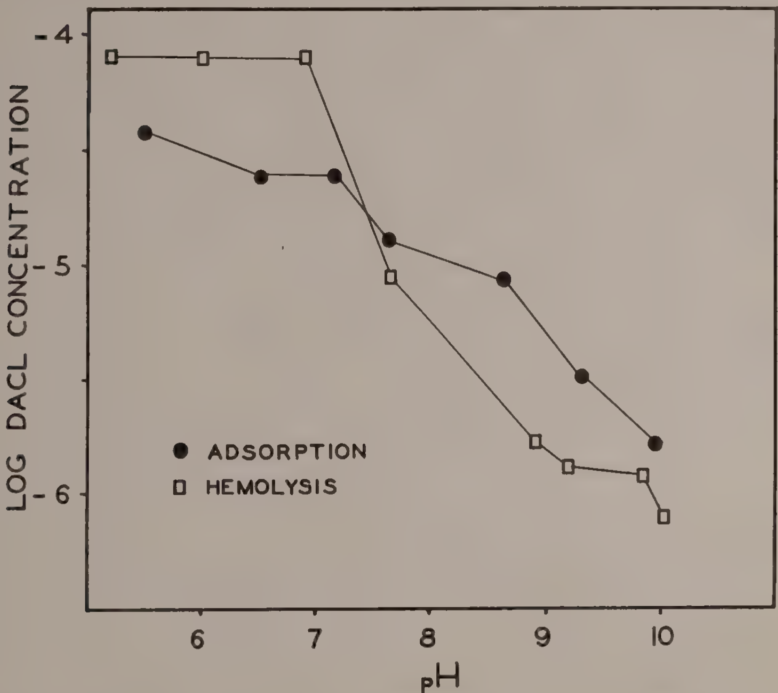

Fig. 7 The effect of pH on the concentration of DACl necessary to produce constant binding, or a constant rate and amount of hemolysis.

that the method of presenting the data used in figure 7 emphasizes the similarity between the two experiments, rather than the differences.

The roughly parallel course of the two curves in figure 7 can be interpreted as indicating a possible direct relation between binding and hemolysis. Despite the formidable difficulties of obtaining smooth curves in such experiments, the

data are in general accord with the hypothesis that alterations of permeability leading to hemolysis are closely related to the quantity of agent bound per cell, provided that hemolysis is sufficiently rapid so that antihemolytic activity is avoided. This behavior of DACl leads to the hope that some of the peculiar effects of pH on oleate and taurocholate hemolysis (Ponder, '48) will be explained along similar lines when binding data for these compounds are available.

Using the extrapolated value of M, the maximum number of binding sites per cell, already obtained, it is of interest to calculate the fraction of the cell surface accounted for when all the sites are occupied by dodecyl ammonium ions. The value so obtained depends on that chosen for the surface area of the erythrocyte. Ponder ('48) estimated this area for the normal biconcave human red cell to be 163 μ^2. On the other hand, dodecyl ammonium ions produce a disc-sphere transformation of the erythrocyte, probably of the sort that occurs with taurocholate (Ponder, '48), i.e., with no change in cell volume; the area of such a spherical human erythrocyte would be about 100 μ^2. Since the area covered by one dodecyl ammonium ion is about 25 Å^2 (Adam, '38), and since an average value of 1.3×10^8 ions per cell is indicated by the corrected y intercepts of figure 3, the fraction of the surface of the biconcave cell covered at maximal adsorption may be estimated to be one-fifth, while the corresponding fraction for the spherical cell is one-third.

Hemolysis data such as those in figure 7, when compared with curves like those in figure 3 show that hemolysis occurs when between 75 and 95% of the sites are occupied. Furthermore, since at high DACl concentrations the cells are spherical, it follows that lysis of the cells could be expected to result from the occupation of between one-third and one-fourth of their surfaces by adsorbed dodecyl ammonium ions. These figures are in rough agreement with Ponder's estimates for the anionic detergents, and certainly agree with his statement that it is not necessary to form a monolayer at the cell surface to produce hemolysis (Ponder, '47). The

situation is apparently different in the case of the anionic photodynamic dye, rose bengal, of which an adsorbed layer three molecules thick is said to be necessary to produce strong hemolysis (Gilbert and Blum, '42).

On the basis of the experiments presented above it is reasonable to suppose that the antibacterial effects of ionic surface-active agents, which are functions of Gram reaction, pH and agent type (Glassman, '48), probably in large part reflect differences in cell-detergent affinity as a function of these parameters. At any event, hemolytic phenomena, where the variables in question have been more carefully isolated and studied independently, seem to be very intimately related to the adsorption of the hemolytic agent.

SUMMARY

1. The reversible adsorption of dodecyl ammonium ions at the erythrocyte surface, as a function of pH and temperature, has been measured by a newly developed method. Increasing the pH strongly increases the cell-detergent affinity; temperature effects are relatively small.

2. A mass action theory of adsorption similar to those used by others for the description of ion-protein interaction has been developed. This theory furnishes a possible explanation of the experimental data in terms of a competition between H ions and dodecyl ammonium ions for at least two kinds of binding sites.

3. A preliminary experiment supporting the theory indicates that the surface of the human erythrocyte binds H ions in appreciable amount.

4. The relation between binding and hemolysis has been examined in the light of these results. Over a pH range from 6.5 to 10.0 with concentrations of the hemolysin varying by a factor of about 100, it has been found that the amount of adsorbed agent required to produce a standard rate of hemolysis varies by no more than a factor of 3 or 4.

5. Calculations show that one-third to one-fourth of the cell surface must be covered by the adsorbed agent to produce hemolysis, but that a monolayer of the hemolysin is not necessary for rapid hemolysis.

ACKNOWLEDGMENT

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PERMEABILITY OF LYMPHOCYTES AND LYMPHOMA CELLS TO POLYHYDRIC ALCOHOLS [1]

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SIX FIGURES

INTRODUCTION

The studies to be presented are a continuation of the investigations reported by Lucké and Parpart ('54). In that report, the permeability of Ehrlich mouse ascites was compared with the erythrocytes of a normal mouse. This work has now been extended to the lymphomas with the intent of comparing these neoplasms with their normal tissue counterparts, the lymphocytes.

MATERIALS AND METHODS

Tumor cells: Three lymphomas were used in these experiments: 1. Murphy-Sturm rat lymphosarcoma ('41), grown in the Wistar rat. 2. Gardner mouse lymphosarcoma, 6C3HED, grown as the ascites in C3H mice ('44). 3. Lewis lymphoma ('53), grown in Lewis rats.[3]

The Murphy-Sturm lymphosarcoma was used at three weeks, the Lewis lymphoma at three to 4 weeks. The Gardner

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[2] Deceased.

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ascites was obtained at 7 days by aspiration of the peritoneum, and used directly, while the solid tumors were prepared as described below.

Cell suspensions were prepared from fresh tumor. The animals were exsanguinated prior to the dissection in order to minimize contamination with blood. Necrosis in these tumors was slight; only clean areas were dissected out for use. The solid tissue was cut fine in 0.1 cc increments of cold Krebs Ringer solution. One drop of heparin-Ringer solution (2 mg/cc) was added to prevent clotting although experience later showed it not to be a necessity. Periodic shaking during the cutting facilitated the removal of the cells from the tissue stroma. The cell suspension was separated from the remaining tissue by aspirating the fluid through a small absorbent cotton pad dropped into the dish containing cells and tissue.[4]

Cell suspensions were kept in closed weighing bottles at refrigerator temperature until used. Time of use in an experiment varied up to five hours after removal from the animal. Although times were kept of the age of the cell suspension for each run in the densimeter, no differences were observed during these periods.

Normal lymphocytes: Lymphocytes were obtained from the axillary, inguinal, and mesenteric lymph nodes, and from the thymus gland. Cell suspensions were prepared in the same manner as the tumor cell suspensions.

Measurements of changes in cell volume: The method employed was that described by Lucké and Parpart ('54). The volumes of cell suspension chosen were such as to give a maximum deflection of the galvanometer when the cells were exposed to solutions of NaCl equal to twice isotonicity. These ranged from 0.05 to 0.5 ml, depending upon the cell suspension.

[4] The authors would like to thank Dr. C. Breedis, Department of Pathology, for demonstrating this technique.

PROTOCOL

In the preliminary studies of Lucké and Parpart ('54), the relative permeability of the tumor cells to polyhydric alcohols was expressed by two measurements: time to a minimum volume and time to $\frac{1}{2}$ equilibrium volume.

The rationale for this approach was based upon the quantitative methods of Jacobs ('33a,b). When one adds a known amount of penetrating solute to an istonic non-penetrating external medium, the cell will shrink to a certain minimum volume after which it will regain its original volume. According to this author, the minimum volume which is obtained will depend upon the ratio, k_2/k_1, where k_2 is the rate constant for the movement of water and k_1 the rate constant for the movement of the penetrating solute.

It is important to remember, however, and Jacobs ('33a) has emphasized this, that k_1 and k_2 are constants whose values "depend upon the units of concentration, volume, and area employed in making the measurements." In developing the relationship between k_2/k_1 (called K in subsequent discussion) and the minimum volume, he has chosen to think of k_2 as "the numerical value which expresses what fraction of the original osmolar content of the cell would pass through an area equal to its initial surface in one minute with a concentration difference on the two sides of the cell membrane equal to the osmolar concentration of the cell; k_1, the permeability constant for water, similarly indicates what fraction of the initial cell volume the amount of water would be that would pass through an area equal to the initial cell surface in one minute with a concentration difference equal to the osmolar concentration of the cell," or its environment with which it is in equilibrium.

The coefficients k_1 and k_2 as determined above will not suffice to compare different cells, for their values are relative to the volumes, surface areas, and osmolar concentrations of the cells. To make comparisons, absolute values for these coefficients are necessary and require knowledge of the volumes, areas, and osmolar concentrations of the cells. Thus

if we let k_{1a} equal the absolute permeability coefficient for the penetrating solute, such that

$$k_{1a} = \text{moles penetrating solute}/\mu^2 \text{ surface area/minute/unit (mol/l) concentration difference}$$

then:

$$k_{1a} = \frac{k_1 \ (V) \ (10^{-15})}{A} \quad \text{where } V = \text{volume in } \mu^3, \ A = \text{area in } \mu^2 \tag{1}$$

and let

$$k_{2a} = \mu^3 \text{ water}/\mu^2 \text{ surface area/min/unit osmotic pressure difference.}$$

then:

$$k_{2a} = \frac{k_2 \ V}{(A) \ (OP)} \quad \text{where OP} = \text{osmotic pressure of the cell.} \tag{2}$$

Now

$$K = k_2/k_1 \tag{3}$$

Solving for k_{1a}

$$k_{1a} = \frac{k_{2a} \ (OP) \ (10^{-15})}{K} \tag{4}$$

Finally, to express k_{1a} in terms of cm/hr we multiply by 6×10^{12}.

Since k_{2a}, the permeability coefficient for water will be determined independently, then a solution for k_{1a} will require only a knowledge of K. Since Jacobs has developed equations for the calculation of K from values for minimum volume ('33b), only this latter information is required. The osmotic pressure of the cell is equal to that of its environment, for in all these treatments, the assumption is made that the cell is in equilibrium with its environment.

For the independent determination of k_{2a}, the permeability coefficient for water, the method of Lucké, Hartline, and McCutcheon, ('31) has been employed. In this method we need to know the successive volumes of the cell as it shrinks in an hypertonic solution. Applying these data in their equation (3), one may then calculate k_{2a}.

Inherent in all these calculations is the underlying assumption that the cells act like perfect osmometers. There-

fore, volumes must be corrected for osmotically inactive material.

To arrive at a permeability coefficient, then, for a penetrating solute under the conditions of our experiments, we must be able to demonstrate the following:

1. A linear relationship between the volume of cell (V), and the reciprocal of the osmolar concentration (1/C). Extrapolation of 1/C to 0 gives a measure of the value for "b", the osmotically inactive material.
2. Some relationship between galvanometer deflection of the densimeter and the volume of the cell, in order to convert the instrumental readings to volume changes of the cell.

Having obtained this information, we may go on to:

3. Calculation of k_{2s}, the permeability coefficient for water.
4. Measurement of the minimum volume of a cell during a shrink-swell experiment, and the expression of this volume as a percent of the initial volume. From this we obtain K.
5. Calculation of k_{1s}, as indicated in equation (4) and conversion to cm/hr.

RESULTS

Figure 1 shows the relationship between the volume of the cell, V, and the reciprocal of the Krebs Ringer solution in equilibrium with it. The volumes were calculated from measurements (with a filar ocular micrometer) of the diameters of these spherical cells. The results represent the averages from three separate experiments. In each experiment, 50 cells, chosen at random were measured to obtain the volumes of the cells in isotonic solutions, while 25 cells were used for volumes of cells in the hypertonic solutions. It should be mentioned that each individual experiment gave linear results, but that separate experiments differed in their initial volumes and "b" values. Where densimeter experiments were run concomitantly, we used the volume data for that experiment to calculate permeability coefficients. Average values as noted in figure 1 were applied to all other experiments. One explanation applicable at least to the Gardner ascites lym-

phoma, for the variation in the isotonic volume of the cells has been reported by Revesz and Klein ('54) who showed an inverse relationship between cell concentration during growth and cell volume.

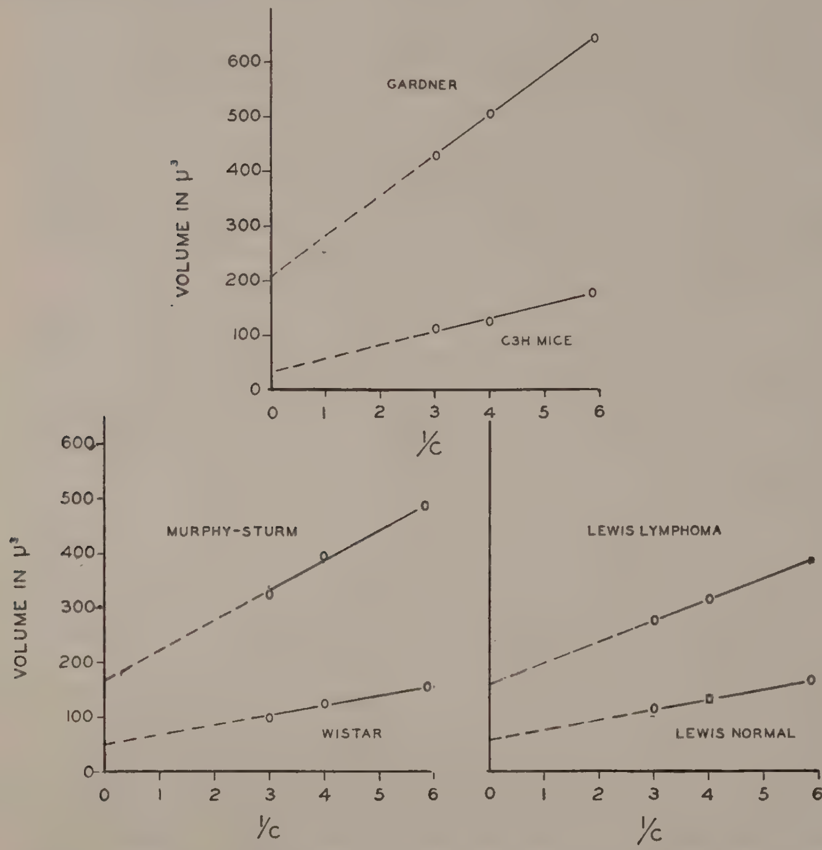

Fig. 1 Relation of cell volume to the reciprocal of the Krebs-Ringer salt solution. Curves extrapolated to $1/C = 0$ for the determination of the volume of osmotically inactive material.

Figure 2 shows the characteristic relation between the deflection of the galvanometer at equilibrium and the concentration of the salt solution. The different slopes of the lines are expressions of the sensitivity of the optical and recording systems and may be varied by moving the camera

or by changing the intensity of incident light. For any given experiment it is important that these remain constant in order to relate deflections to volumes of cells.

Since galvanometer deflection is a function of concentration and volume is a function of concentration, then deflection is some function of volume. Figure 3 illustrates the graphical

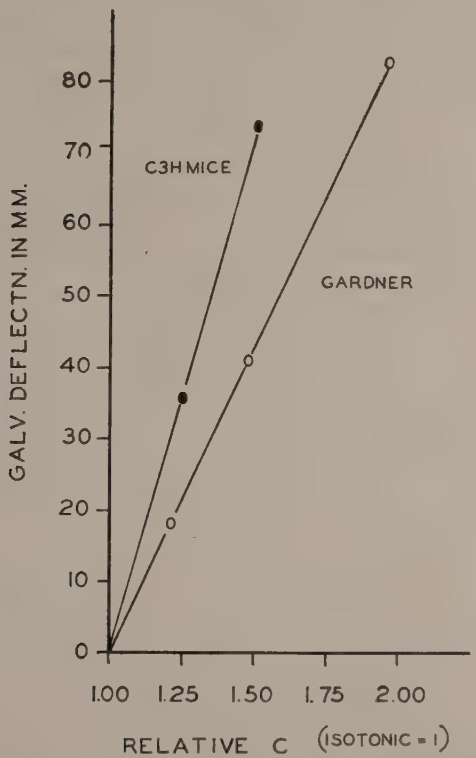

Fig. 2 Calibration curve showing the relation between deflection of the galvanometer and the shrinkage of cells in hypertonic solutions of Krebs-Ringer.

method we have employed to convert deflection into volume. From a given deflection one reads off the relative concentration (isotonic = 1). The hyperbolic curve defines the relation between relative concentration and the reciprocal of the actual salt concentration. From this curve one obtains a value of 1/C. From the relationship of V-b to 1/C, one reads

off the value for the volume of the cell, corrected for osmotically inactive material.

Figure 4 illustrates the use of the equation of Lucké, Hartline, and McCutcheon, for the calculation of the permeability coefficient for water (k_{2a}). In contrast to Arbacia eggs,

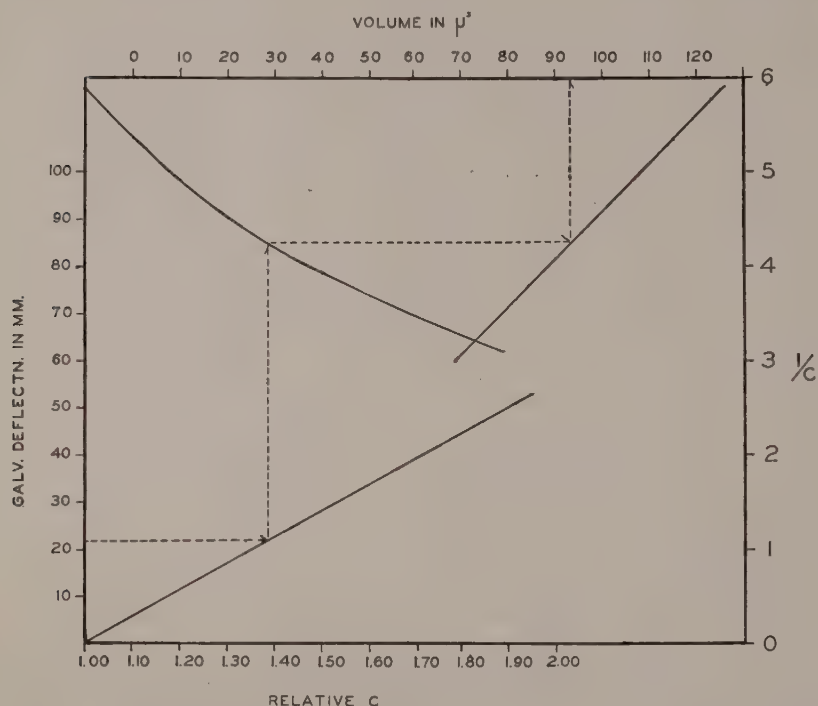

Fig. 3 Graphical conversion of galvanometer deflections to actual volume changes in intracellular water.

where the relationship is shown to be linear over most of the time course, such has not been the case for these cells. In our investigations, the coefficient has slowly decreased along the time course toward equilibrium. We have arbitrarily chosen the time interval between the first and the fifth second, where linearity is best maintained, for the calculation of the permeability coefficient for water, k_2.

In table 1 we have collected our measurements on the rate of movement of water out of the cell, under a driving force of twice osmolar concentration.

In table 2 and figure 5 we have gathered our results on the permeability of normal cells and their neoplastic counterparts to a series of homologous polyhydric alcohols, and to glycerol.

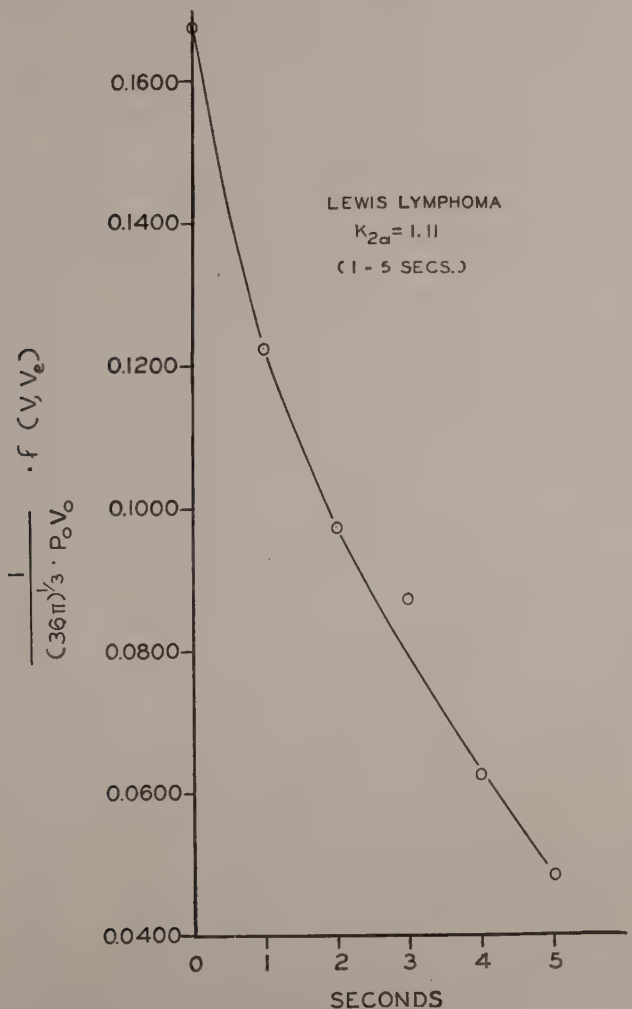

Fig. 4 Illustration of the application of the equation of Lucké et al. for the determination of the exosmotic permeability coefficient for water.

DISCUSSION

Summing up the results of our investigations on this series of homologous polyhydric alcohols, and glycerol has provided us with certain definite conclusions. We have established, to a first approximation, permeability coefficients for the rat and mouse lymphocytes and their neoplastic correlates on an absolute basis, expressed in terms which permit their

TABLE 1

Permeability coefficients for water (exosmosis). Driving force = 2 O.P.

CELL TYPE	AVG. VOLUME IN μ^3 (UNCORRECTED FOR OSMOTICALLY INACTIVE MATERIAL)	"b" VALUE (%)	$P = \mu^3/\mu^2$/MIN/OP DIFF. (ONE–FIVE SECONDS)	SIGNIFICANCE
Lewis rat lymphocytes	166 ± 8.2	36	0.84 ± 0.11	(4) n.s. $P = 0.2$
Lewis rat lymphoma	389 ± 30	40	0.97 ± 0.04	(6)
Wistar rat lymphocytes	157 ± 3.5	26	0.83 ± 0.02	(6) n.s. $P = 0.15$
Murphy-Sturm lymphoma	483 ± 43	36	0.93 ± 0.06	(6)
C3H Mouse lymphocytes	181 ± 12	21	0.92 ± 0.06	(6) n.s. $P = 0.08$
Gardner mouse ascites	644 ± 37	31	0.78 ± 0.04	(6)

n.s. = not significant.
( ) = degrees of freedom, d.f.

comparison with other cells. Thus, with respect to exosmosis of water, these cells lie between the Arbacia egg ($0.1\ \mu^3/\mu^2$/min/OP difference) and the red blood cell ($3\ \mu^3/\mu^2$/min/OP difference). See also Lucké, Hartline, Ricca ('39). These values also compare favorably with those obtained by Brues and Masters ('36) for tissue culture cells. So far as penetrating solutes are concerned, comparisons may be made with some values of other cells, as tabulated by Jacobs ('52).

TABLE 2

Permeability of normal cells and their neoplastic counterparts to a series of homologous polyhydric alcohols

CELL TYPE	ETHYLENE GLYCOL			DIETHYLENE GLYCOL			TRIETHYLENE GLYCOL			GLYCEROL		
	$P \times 10^4$ (cm/hr.)	$\pm$ S.E. mean	Significance	$P \times 10^4$ (cm/hr.)	$\pm$ S.E. mean	Significance	$P \times 10^4$ (cm/hr.)	$\pm$ S.E. mean	Significance	$P \times 10^4$ (cm/hr.)	$\pm$ S.E. mean	Significance
Lewis rat lymphocytes	679	123	n.s.	478	144	n.s.	277	95	n.s.	78	14	n.s.
Lewis rat lymphoma	1207	207	(4)	620	119	(4)	250	45	(6)	65	37	(4)
Wistar rat lymphocytes	862	78	n.s.	413	40	n.s.	204	86	n.s.	41	4	n.s.
Murphy-Sturm lymphoma	619	180	(4)	239	66	(4)	160	78	(3)	117	59	(2)
C3H Mouse lymphocytes	758	176	n.s.	530	66	n.s.	181	25	n.s.	81	11	n.s.
Gardner mouse ascites	803	188	(5)	471	73	(5)	202	52	(6)	77	46	(4)

n.s. = not significant.
( ) = degrees of freedom, d.f.

We can further conclude that within the limits of this investigation we have not found any statistically significant difference between the normal lymphocyte and the lymphoma cell with regard to the exosmotic permeability to water or

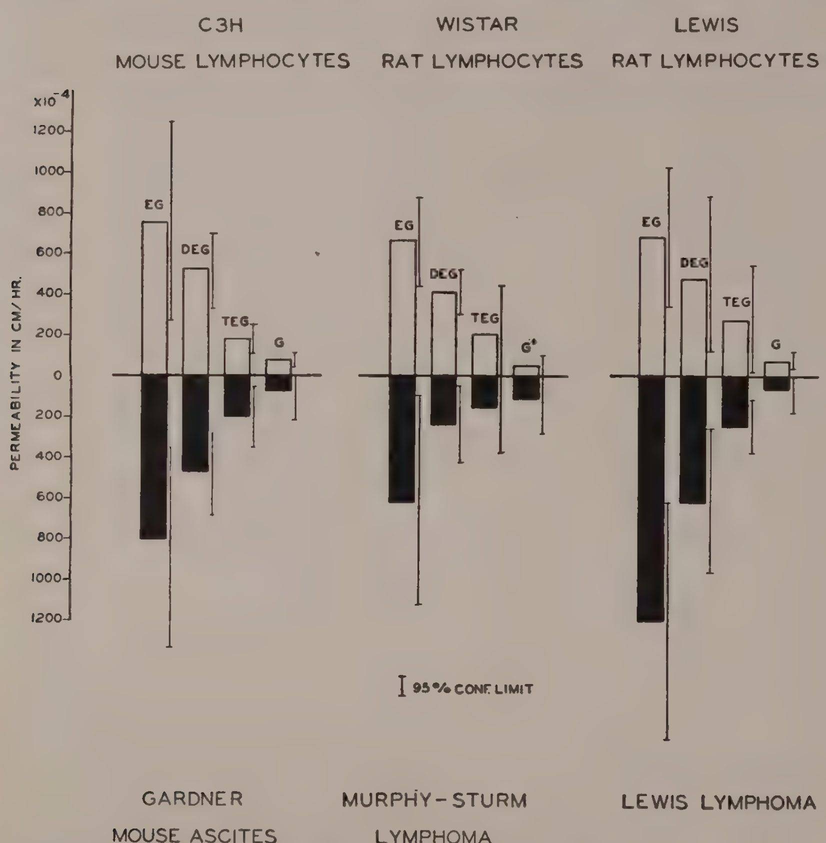

Fig. 5 Permeability coefficients of normal and neoplastic cells to several penetrating solutes.

with respect to the rate of penetration of polyhydric alcohols. We hasten to add, however, that for *any given cell* there is a decreasing rate of permeability with increasing molecular volume. Although, it is true that by grouping the results of several experiments on different cell suspensions of a given cell type the large variations tend to obscure this observation

from a statistical point of view, yet it has been our observation that for any given cell suspension, the order of penetration is ethylene glycol > diethylene glycol > triethylene glycol > glycerol.

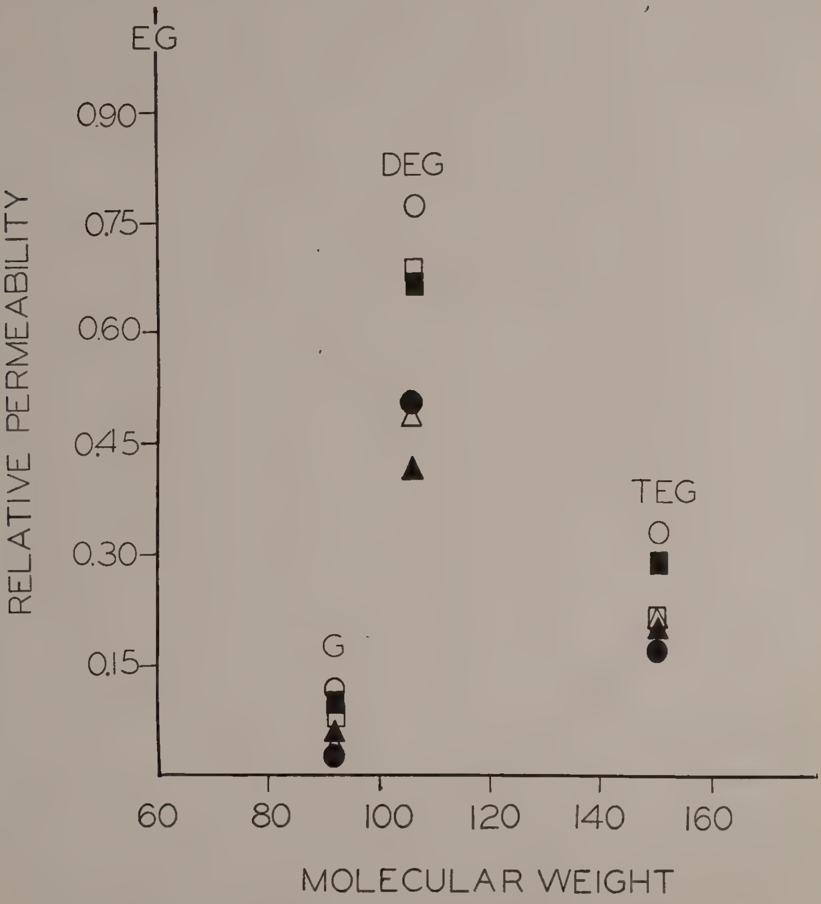

Fig. 6 Relation of molecular weight to relative permeability of several penetrating solutes. Permeability of ethylene glycol taken as equal to 1.

To demonstrate this quantitatively, we have resorted to a *relative comparison* of the permeability of these compounds *within* each cell group, setting the permeability coefficient for ethylene glycol equal to 1. Table 3 presents the data as

TABLE 3

Relative permeability of normal and neoplastic cells with permeability P, to ethylene glycol taken as equal to 1

CELL TYPE	ETHYLENE GLYCOL	DIETHYLENE GLYCOL			TRIETHYLENE GLYCOL			GLYCEROL	
	P	P	± S.E. mean		P	± S.E. mean		P	± S.E. mean
Lewis rat lymphocytes	1	0.77 ±	0.203	← n.s. →	0.33 ±	0.066	← s →	0.096 ±	0.023
		↕ n.s.			↕ n.s.			↕ n.s.	
Lewis rat lymphoma	1	0.51 ±	0.043	← s →	0.17 ±	0.038	← s →	0.026 ±	0.009
Wistar rat lymphocytes	1	0.48 ±	0.031	← s →	0.22 ±	0.087	← n.s. →	0.043 ±	0.011
		↕ n.s.			↕ n.s.			↕ n.s.	
Murphy-Sturm lymphoma	1	0.41 ±	0.042	← s →	0.20 ±	0.061	← n.s. →	0.056 ±	....
C3H Mouse lymphocytes	1	0.68 ±	0.512	← n.s. →	0.22 ±	0.031	← s →	0.071 ±	0.027
		↕ n.s.			↕ n.s.			↕ n.s.	
Gardner mouse ascites	1	0.67 ±	0.139	← s →	0.29 ±	0.057	← s →	0.090 ±	0.035

s = significant; $P < 0.05$.
n.s. = not significant.

analyzed statistically while figure 6 gives a graphic representation.

When handled in this fashion, the data still does not offer evidence for significant differences among cell types, even when one compares one cell to another with respect to the relative decrease in permeability from one solute to the next. However, figure 6 does point up the relation between the molecular weight of these compounds (which does not differ significantly from the molecular volume) and their relative rates of penetration.

This type of data adds more evidence to the view that the spatial configuration of the molecule influences its ability to penetrate the membrane. Thus, for example, glycerol is much slower in its rate of penetration than ethylene glycol, even though their molecular weights do not differ markedly. The concept of the inhibitory property of the —OH group on the rate of penetration dates back to Overton's classical work. Other evidence may be found in the work of Höber and Orskov ('33), and Jacobs, Glassman, and Parpart ('35). See also Davson and Danielli ('43).

In the light of this evidence then, we may deduce that one property of the membrane of these cells is its sieve-like qualities. (cf. Collander and Barlund ('33), and Jacobs ('52). This appears to be true both of normal and neoplastic cells. However, differences between these cells may become evident as one probes deeper into the nature of the interstices of this sieve. For example, Magath ('32) has summed up indirect evidence for enhanced electrolyte permeability in neoplasms. Notice had been taken of the lower impedance of neoplasms to alternating current. Swelling of tumor tissue was shown to occur in isosmotic buffered acid solutions, a phenomenon which, in red blood cells, requires a permeability to anions. This swelling is slowed considerably if the tissue is previously soaked in Ca solutions. In turn, Ca ion has been shown to be diminished in tumor tissue, according to Coman ('47), who has correlated this deficit with a diminished adhesiveness of tumor cells to each other.

We find ourselves then, in this first stage of a physiological analysis of the tumor cell membrane, at a level which exhibits gross porosity. Only subsequent work will elaborate the niceties which may permit a discrimination between the membrane properties of normal and neoplastic cells, if such is possible.

SUMMARY

1. Permeability coefficients of three different lymphoma cells and their normal counterparts were determined for a series of polyhydric alcohols and glycerol and for the outward movement of water.

2. No significant differences in rates of penetration of the solutes tested could be found when cell types were compared.

3. For each cell type, the rates of penetration of the solutes tested could be arranged in the order: ethylene glycol > diethylene glycol > triethylene glycol > glycerol.

4 Results would indicate that the membranes of the normal and neoplastic cells tested have sieve-like properties of the same degree of porosity.

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THE SEPARATION OF CATION EXCHANGE AND GLYCOLYSIS IN HUMAN RED CELLS EXPOSED TO NON-IONIZING RADIATIONS [1]

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FOUR FIGURES

The association of glycolysis with the maintenance of normal Na and K gradients in mammalian erythrocytes under *in vitro* conditions has been observed in experiments in which glycolysis has been interfered with by metabolic poisons (Harris, '41; Maizels, '51; Green and Parpart, '53) or by cold (Harris, '41; Ponder, '51; Maizels, '51). Irradiation of red cells with X rays was shown by Sheppard and Stewart ('52) to accelerate the exchange of Na and K without interfering with glycolysis. The hemolytic effects of non-ionizing radiations have been known for some time (Hasselbach, '09; Sonne, '29; Lepeschkin, '31; Leu, Wilbrandt and Liechti, '42) and in recent years the mechanism of action has been associated with an increased Na and K permeability (Leu et al., '42). None of the earlier studies was concerned with the relation between cation exchange and glycolysis.

The object of the present paper is to report the results of irradiation studies of human red cells using non-ionizing radiations extending from the ultraviolet through the visible. The results show that such radiations accelerate cation exchange without inhibiting glycolysis and are interpreted to mean that the cell surface is important in cation regulation under these experimental conditions.

[1] This work was performed in the laboratory of Professor A. C. Giese of Stanford University during the tenure of a Ford Foundation Fellowship.

METHODS

Human blood was collected by veni-puncture in a sterile syringe. The blood was defibrinated, filtered through gauze to remove small clots and either used immediately or refrigerated until used, always within 4 hours of collection. Before use, the blood was centrifuged, the serum removed and the cells washed three times in 1% NaCl-PO_4 (Parpart and Green, '53) at pH 7.35. Cell concentration proved to be an important variable in these studies and the concentrations finally arrived at (approximately 5%) represented the most dilute suspensions which could be conveniently analyzed and yet were of sufficient concentration to show appreciable glucose utilization within the time limits of the experiment. Glucose was added to the cell suspensions in concentrations of 1 mg per milliliter of suspension.

The experiments were performed in quartz or glass Warburg vessels mounted in the conventional manner in a water bath maintained at $37 \pm 1°C$. Each vessel contained 5 ml of red cell suspension. This quantity of suspension was sufficiently small and dilute to prevent the cells from settling at ordinary shaking velocities. The cells were agitated continuously during the 12 hours of each experiment.

For convenience the radiation sources used are referred to as emitting ultraviolet or visible radiations. Since the only filter used was pyrex glass (as containers) it is recognized that the ultraviolet source emitted light of wavelengths longer than 3100 A and that the visible light source emitted radiations of at least 3100 A which could be transmitted through the glass. Thus, there was an area of overlap in the two radiation sources and the effects observed by the so-called visible light may have been owing chiefly to the longer UV wavelengths.

Irradiation with ultraviolet light was accomplished in the following manner: A specially designed "Steri-lamp" (low pressure mercury lamp in quartz) was mounted in the water bath between the Warburg vessels and one side of the bath

in such a manner that the lamp was approximately 1 cm from the bottom and side of the vessels. Three quartz vessels containing cell suspensions were placed directly in the light of this lamp which emitted at the center of the Warburg vessels approximately 123 ergs/sec/mm^2 of light energy, an estimated 85% of which was at 2537 A°. Cells were exposed to this amount of radiation for one hour making a total of 4.4×10^5 ergs/mm^2 of radiation received in the vicinity of these cells. Three control suspensions were placed in glass Warburg vessels mounted in the water bath on the same side as those exposed to the steri-lamp but not directly in its radiation path. Cell suspension samples were taken before irradiation, at the end of irradiation, 5 and 11 hours after irradiation from one irradiated and one non-irradiated vessel. These samples were analyzed for glucose changes, Na, K, pH, hemolysis and cell volume change (Parpart and Green, '53). Glucose analyses were performed by the method of Nelson ('44) as modified by Somogi ('52). Gaseous exchange was followed for the 12 hours of the experiment manometrically.

Irradiation with visible light was performed in essentially the same experimental set up except that the light source was mounted outside of the bath under a glass shelf immediately above which the Warburg vessels were mounted in the bath. The light source for each irradiated vessel was a G-E CH 4 spot lamp emitting approximately 19,000 foot candles (approximately 3×105 ergs/sec/mm^2) at the bottom of the Warburg vessels. A petri dish filled with water was mounted directly over each lamp to prevent fracture of the glass shelf bottom of the water bath. Control Warburg vessels not to be irradiated were wrapped carefully in aluminum foil and mounted in the bath out of the beam of the spot lights. For visible light irradiation all Warburg vessels were of glass. The temperature inside the illuminated Warburg vessels was checked with a small thermometer mounted in the side arm of the vessel and found not to rise more than 0.5°C. above that of the water bath. Three non-irradiated

and three irradiated vessels were mounted on one side of the water bath. The experimental vessels were irradiated for 4 hours. Suspension samples were removed from control and experimental vessels at 4, 8 and 12 hours and the same analyses performed as in the case of the ultraviolet irradiated cells. Gaseous exchange was determined manometrically over the 12 hour period.

RESULTS

Ultraviolet irradiation

Typical curves for the Na-K exchange occurring in human red cells exposed to one hour of irradiation with dosages of

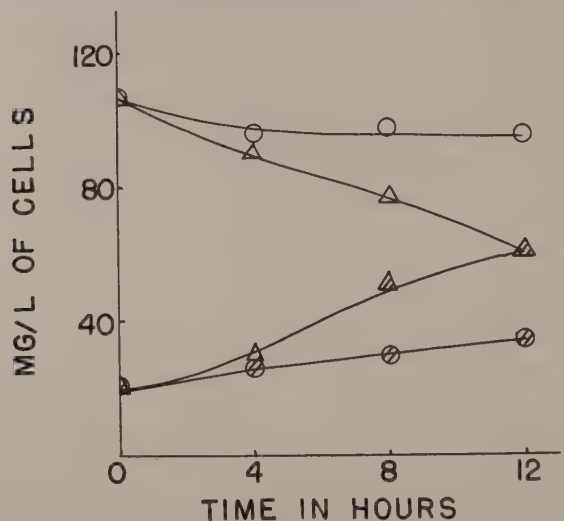

Fig. 1 One hour ultraviolet irradiation of human red cells. Plot of K loss and Na again in mg/l of cells against time including the irradiation period. ○ = K loss, non-irradiated cells; ● Na gain, non-irradiated cells; △ K loss, irradiated cells; ▲ Na gain, irradiated cells.

ultraviolet light are shown in figure 1. During a 12 hour period non-irradiated cells lost 15% and irradiated cells lost 38% of their K. The irradiated cells gained over 5 times as much Na as did the non-irradiated. In this experiment and in general this was found in other experiments, there was

nearly an equivalent exchange of Na for K. No significance is attached to the fact that most of the K lost and Na gained occurred in the first 6 hours. This was not commonly observed. And indeed with three hours of ultraviolet radiation the exchange proceeded to diffusion equilibrium at the end of the radiation.

Figure 2 is a plot of the supernatant glucose concentration against time for cells irradiated with ultraviolet for one hour

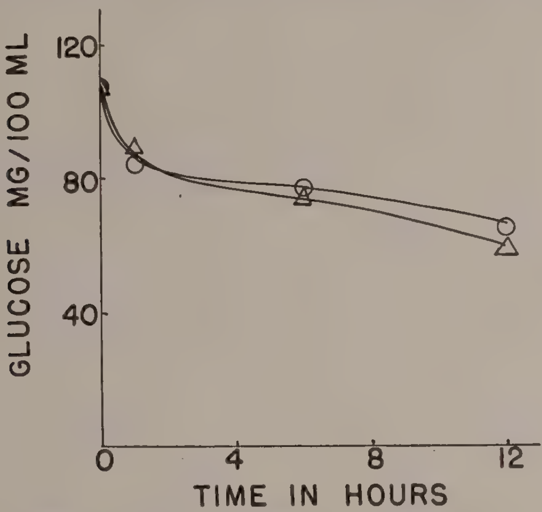

Fig. 2 One hour ultraviolet irradiation of human red cells. Plot of glucose concentration in cell suspension against time including the irradiation period. ○ = Non-irradiated suspension; △ = irradiated suspension.

and non-irradiated cells. This plot shows the disappearance of glucose from the medium and while disappearance alone does not mean consumption, a simultaneous drop in the pH of the medium (table 1) strengthens the assumption that the cell is utilizing this material. Of particular interest is the parallel uptake of glucose by both irradiated and non-irradiated cells. This parallelism is further shown by the pH of these suspensions in table 1 and the presumption is that irradiated and control cells are transporting and utilizing glucose at the same rate. By contrast the irradiated cells

have a K-Na exchange rate which is appreciably greater than that observed in the controls.

Table 1 also lists the per cent of hemolysis found in irradiated and non-irradiated suspensions during the experiment. The amount of hemolysis in irradiated cells is consistently higher than in the control cells amounting at the end of 12 hours (11 hrs. after irradiation) to over 9 times as much. This finding is also supported in part by the consistently greater amount of swelling occurring in irradiated cells as shown by table 1.

TABLE 1

1 Hour ultraviolet irradiation

TREATMENT	pH	HEMOLYSIS %	VOLUME CHANGE AND INITIAL HB	GAS LIBERATED (+) OR TAKEN UP (—) μl/hr.
Before irradiation	7.46	0.53	100	..
End of irradiation (glass)	7.44	0.70	100	+1.3
End of irradiation (quartz)	7.44	0.70	92.4	—8.5
5 Hrs. after irradiation (glass)	7.28	1.05	99	—3.3
5 Hrs. after irradiation (quartz)	7.28	2.46	89.5	—3.1
11 Hrs. after irradiation (glass)	7.18	1.40	100	—0.8
11 Hrs. after irradiation (quartz)	7.18	12.80	88.5	—3.0

The gas uptake as shown by table 1 is greater in irradiated than non-irradiated cells. The magnitude of uptake in irradiated cells is generally greatest during the radiation period.

Visible light irradiation

Figure 3 is a plot of the Na-K exchange with time when cells are irradiated with strong visible light for a 4 hour period. This graph shows as did figure 1 that irradiated cells lose K and gain Na at an appreciably faster rate than the non-irradiated cells. Figure 3 further shows that the accelerated cation exchange is essentially an equivalent exchange of Na for K as was the case with ultraviolet radiation.

The changes in supernatant glucose concentration with time in irradiated and control cells are plotted in figure 4.

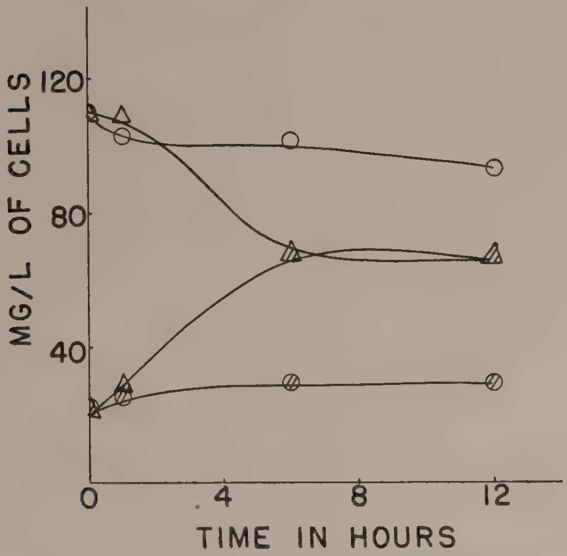

Fig. 3 Four hours visible light irradiation of human red cells. Plot of K loss and Na gain in mg/1 of cells against time including the irradiation period. ○ = K loss, non-irradiated cells; ● = Na gain, non-irradiated cells; △ K loss, irradiated cells; ▲ Na gain, irradiated cells.

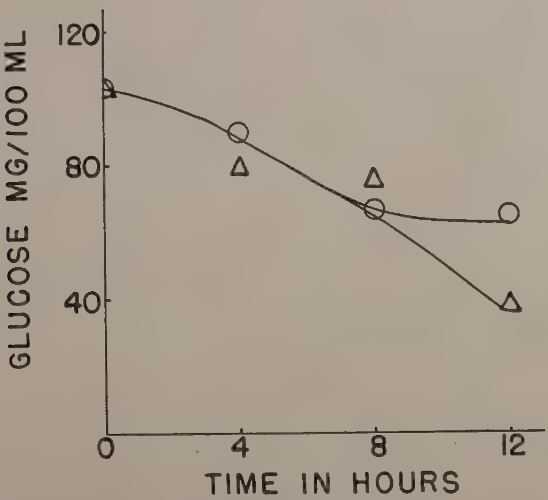

Fig. 4 Four hours visible light irradiation of human red cells; Plot of glucose concentration in the cell suspension against time including the irradiated period. ○ = Non-irradiated suspension; △ = irradiated suspension.

The rate of disappearance of glucose from the suspension media is approximately the same with the possible exception of the greater uptake by irradiated cells between 8 and 12 hours. This parallelism is supported by the pH data of table 2. This table also shows that irradiation with visible light increases the amount of hemolysis although not to the same degree as was observed with ultraviolet irradiation. Irradiated cells swell to a larger volume than do non-irradiated cells.

TABLE 2

4 Hours irradiation with visible light

TREATMENT	pH	HEMOLYSIS %	VOLUME CHANGE AND INITIAL HB	GAS LIBERATED (+) OR TAKEN UP (—) μl/hr.
Before irradiation	7.40	0.36	100	. .
End of irradiation (dark)	7.55	0.50	101.2	+ 6.4
End of irradiation (light)	7.57	1.23	100	— 0.4
4 Hrs. after irradiation (dark)	7.42	0.58	100	+ 3.8
4 Hrs. after irradiation (light)	7.41	1.67	92.8	— 10.8
8 Hrs. after irradiation (dark)	7.38	0.72	98.5	+ 1.6
8 Hrs. after irradiation (light)	7.38	3.34	94.4	— 9.4

The gas exchange data of table 2 reveal a small loss of gas from non-irradiated cells which represents possibly a reduction of hemoglobin while the irradiated suspensions show an uptake of gas.

DISCUSSION

Cation exchange in mammalian erythrocytes *in vitro* is one of the most characteristic features of pre-hemolytic changes in these cells (Davson and Danielli, '38). It occurs with temperature, salt and hydrogen ion change (Parpart et al., '47), with lytic agents (Ponder, '47, '48; Parpart and Green, '51), narcotics (Grieg et al., '53; Green and Wazeter, '53) with glucose and metabolic inhibitors (Green and Parpart, '53), with X-irradiation (Sheppard and Stewart, '52) and as shown by this report with ultraviolet and visible light. In potassium-rich red cells, two types of exchanges can be recog-

nized. One in which there is an equivalent exchange of Na for K, and one in which there is an unequal exchange. In general the former occurs under those conditions in which glycolysis is not interfered with; the latter under conditions in which glycolysis is inhibited. This generalization implies that agents or conditions which modify the red cell surface but not the metabolism of the cell produce equivalent cation exchange. Those which produce unequal exchange must interfere with the cell metabolism or with both the surface and metabolism. Equivalent exchange of cations probably means that diffusion processes are predominantly regulated by the cell surface while unequal exchange strongly suggests the operation of dual mechanisms for the control of Na and K.

The present work supports this scheme by showing that cells exposed to both ultraviolet and visible light continue to utilize glucose at the same rate as non-irradiated cells, yet exchange K and Na at a much accelerated but equivalent rate. Sheppard and Stewart ('52) obtained a similar result with cells exposed to X rays.

Sonne ('29) published an action spectrum for red cell hemolysis by ultraviolet light and found that the shorter wavelengths were most effective in producing hemolysis. No attempt was made in the present study to determine the wavelengths effective in cation exchange. And indeed the exchange observed with visible light may be owing to the longer wavelengths of ultraviolet for at least those down to 3100° A were transmitted by the glass vessels. Tables 1 and 2 show that the greatest amount of hemolysis occurred with ultraviolet light. And assuming that the effect wrought by the visible light was by the longer wavelengths one has a very crude confirmation of Sonne's results provided that the Steri-lamp emitted most of its radiation at a wavelength of 2537 A. Further, if the hemolysis is to be attributed to a change in the cell surface, increasing its cation permeability, which seems probable (Parpart and Green, '51) the action spectrum of Sonne for hemolysis may be also one for cation exchange. Lepeschkin's ('31) studies of the hemolytic action

of sunlight find some confirmation in the experiments here. He was able by filters to show that the longer ultraviolet wavelengths transmitted by glass and the shorter visible wavelengths were more effective in producing hemolysis than were the longer visible wavelengths. According to him the action of the light was to modify certain compounds in the cell protoplasm which in turn brought about the hemolysis.

Blum ('41) pointed out that photodynamic hemolysis was dependent upon the presence of oxygen in the suspension medium and that the hemolysis produced by visible light did not occur in the absence of oxygen. He attributed the oxygen dependence in this latter instance to a photodynamic action in which a small amount of protoporphyrin in the cell acts as a photosensitizer. The modification of cation permeability observed here appears to be centered in the cell surface but whether the lipid or protein components are disoriented cannot as yet be distinguished with certainty. The consistent uptake of gas, probably oxygen, by irradiated cells which are suspended in an atmosphere which maintains the cells completely oxygenated, indicates that oxidation of some surface component is probably occurring. The lipid components of the plasma membrane may be involved (Jacobson and Giese, '53) and a change in their structural relations could modify the cell permeability (Parpart and Ballentine, '52). However, Blum and Cook ('54) have obtained some evidence that ultraviolet irradiation affects the protein components of the cell surface and in these experiments in which uncertainty exists as to the effective wavelengths change in proteins may be responsible for the cation shifts observed.

Further work is needed to establish beyond question which surface components are involved. The solution of this problem should provide new insight into the physical factors regulating cellular cations.

SUMMARY

Washed human red cells suspended in phosphate buffered, NaCl solutions were exposed to either visible or ultraviolet

light for varying lengths of time. Cation exchange, glucose uptake, cell volume change and gaseous uptake were measured up to 12 hours from the beginning of irradiation.

Both ultraviolet and visible radiations accelerated equivalent sodium and potassium exchange while glucose uptake and utilization continued at non-irradiated rates. Irradiation was accompanied by an uptake of gas, probably oxygen and an increase in cell volume.

The implication of these findings for the regulation of cations in erythrocytes was discussed.

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THE RELATION BETWEEN RATE AND AFFINITY IN CARRIER TRANSPORTS[1]

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THREE FIGURES

The penetration of sugars across the human red cell membrane appears to be a complex process involving elements different from simple physical membrane diffusion. An interpretation assuming a carrier mechanism has been suggested by Le Fèvre. It was shown by this author as well as by Wilbrandt and Rosenberg and by Widdas that the penetration kinetics agree with the kinetics of a carrier transport under certain conditions.

The present paper deals with the dependence of the transport rate on the affinity to the components of the assumed carrier system (carrier and enzymes).

It was assumed by Rosenberg and Wilbrandt that the two reactions forming and breaking the substrate carrier complex are catalyzed by enzymes. The total transport resistance, under this condition, was shown to be composed of three additive partial resistances: two reaction resistances, related to the two enzyme reactions and one diffusion resistance related to the diffusion of the substrate carrier complex across the membrane.

Whether one of these resistances is rate limiting could not be decided from the kinetics. The kinetics type which agreed best with observation (type E) can emerge under the conditions of a limiting diffusion resistance as well as a limiting reaction resistance.

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With respect to the relation between rate of transport and affinity these two conditions do not differ widely. The feature dealt with in this paper qualitatively is common to both. Quantitative evaluations are not attempted at present. For the sake of simplicity therefore a limting diffusion resistance will be assumed in the following discussion, in which case the equations to be used are somewhat simpler.

If the diffusion resistance is rate limiting the rate of transport is given by the equation

$$v = D'C_T \frac{K_r\ (S_I - S_{II})}{(S_I + K_r)\ (S_{II} + K_r)} \qquad (1)$$

in which

v = rate of penetration (amount of substrate penetrating per unit time and unit surface area)

D' = diffusion constant of the carrier substrate complex in the membrane divided by the thickness of the membrane

C_T = total carrier concentration

K_r = dissociation constant of the substrate carrier complex

$\left.\begin{matrix} S_I \\ S_{II} \end{matrix}\right\}$ = substrate concentrations on the two sides of the membrane.

This equation implies a peculiar relation between rate of transport and affinity. The relation depends on the ratios $\frac{S_I}{K_r}$ and $\frac{S_{II}}{K_r}$, in other words on whether the carrier is below half saturation (S_I, $S_{II} < K_r$) or near saturation (S_I, $S_{II} > K_r$). With the carrier below half saturation the rate of transport ceteris paribus is proportional to the affinity (inversely proportional to the dissociation constant K_r):

$$v = \frac{D'C_T}{K_r}\ (S_I - S_{II}) \qquad (2)$$

If the carrier is near saturation the rate is inversely proportional to the affinity (proportional to the dissociation constant K_r):

$$v = D'C_T K_r \frac{(S_I - S_{II})}{S_I S_{II}} \qquad (3)$$

Given a series of compounds using the same carrier, the order of penetration rates thus should be the order of affinities in the range of low substrate concentrations, whereas with high concentrations the order should be reversed.

This corollary can be tested experimentally. Le Fèvre has given the following series of affinities as concluded from competition experiments: glucose, mannose, xylose, galactose, arabinose, sorbose, fructose. The transport rates can be determined with the aid of osmotic methods over a concentration range from about 0.015 molar to 1.5 molar. K_r according to Widdas is about 0.010 molar for glucose and higher for the other sugars.

In the experiments to be reported here determinations of the transport rates of the sugars mentioned above were carried out over a wide range of concentrations. They appear to bear out the predictions derived from the assumption of a carrier system. Bearings on observations from other transport systems will be dealt with in the discussion.

METHODS

Human red cells were used. Blood was withdrawn by venous puncture, defibrinated, filtered through gauze and cold stored until it was used for the experiments (not more than three days).

The transport rates were determined by osmotic methods (Wilbrandt, '38, '47). In the range of high concentrations the indirect method was used, for low concentrations the direct method.

In the indirect method one volume of blood was suspended in 10 volumes of isotonic NaCl solution buffered with m/60 sodium phosphate to pH 7.4 and containing one of the sugars in the desired concentration. The suspensions were kept at 37°C.; samples were taken at intervals of time and used for the determination of osmotic fragility (0.5 ml suspension + 10 ml hypotonic NaCl solutions, again phosphate buffered, pH 7.4). The amount of sugar that had entered into the cells was computed from the shift of the fragility curves as described previously.

In the experiments using the direct method 50 µl of blood suspension (1 volume blood + 10 volumes isotonic NaCl solution phosphate buffered, pH 7.4) were mixed in a cuvette with

0.7 ml of hypotonic NaCl solution (of a tonicity causing just beginning hemolysis). After one minute 0.3 ml glucose solution of the same tonicity were added, the suspension thoroughly mixed and its light transmission followed by means of a photoelectric cell (direct opacimetry). From the increase in light transmittance and the curve of osmotic fragility (used as volume calibration curve) the time course of sugar penetration was computed as described previously.

The sugars used were d(+)-mannose "Roche," d(+)-galactose "Roche," l(+)-arabinose "Roche," l(—)-sorbose "Roche," d(+)-xylose, d(—)-fructose "Sandoz" and d(+)-glucose "Sandoz." All experiments were carried out at 37°C.

RESULTS

In figure 1 experiments in the range of low concentrations (0.03 mol) are shown for glucose, mannose, galactose, arabinose and sorbose. Glucose and mannose show the most rapid penetration, galactose is slower, arabinose and sorbose slower still. The order with respect to the rate of penetration thus appears to follow the order of affinities. Experiments with a concentration of 1.5 molar are shown in figure 2. In this series glucose is the slowest sugar, sorbose the fastest. The order is completely reversed. Figure 3 shows the relative rates of penetration R (the rate of glucose taken arbitrarily as equal to one) for the same sugars in three concentrations. The transition appears to be gradual.

With respect to the two other sugars xylose and fructose a complete reversal of the order was not achieved by raising the concentration from 0.03 molar to 1.5 molar. Qualitatively however their behaviour also agrees with prediction. As shown in table 1 fructose in the lowest concentrations used is the slowest of all sugars. With rising concentrations its position gradually shifts towards the faster end of the series. It appears possible that it would reach the top of the series in a concentration range above 1.5 molar which however cannot well be used experimentally. Xylose in low concentrations ranges among the fastest sugars. With rising concentrations

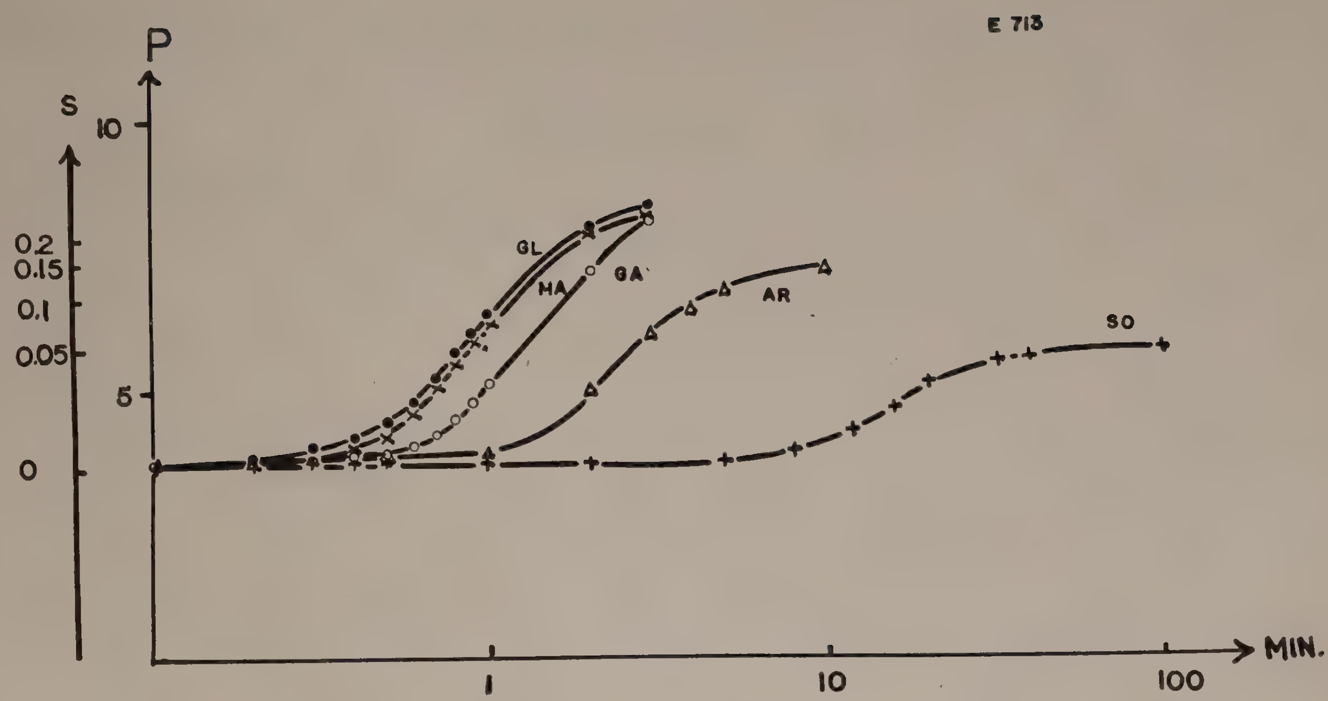

Fig. 1 Penetration of six sugars into human red cells from 0.03 molar solutions (in 0.07 molar NaCl) at 37°C. Direct method. Ordinate: S = amount of sugar having penetrated the cells (cell units; isotonicity = unit concentration, cell volume = unit volume). P = photometric reading. Abscissa: time in minutes.

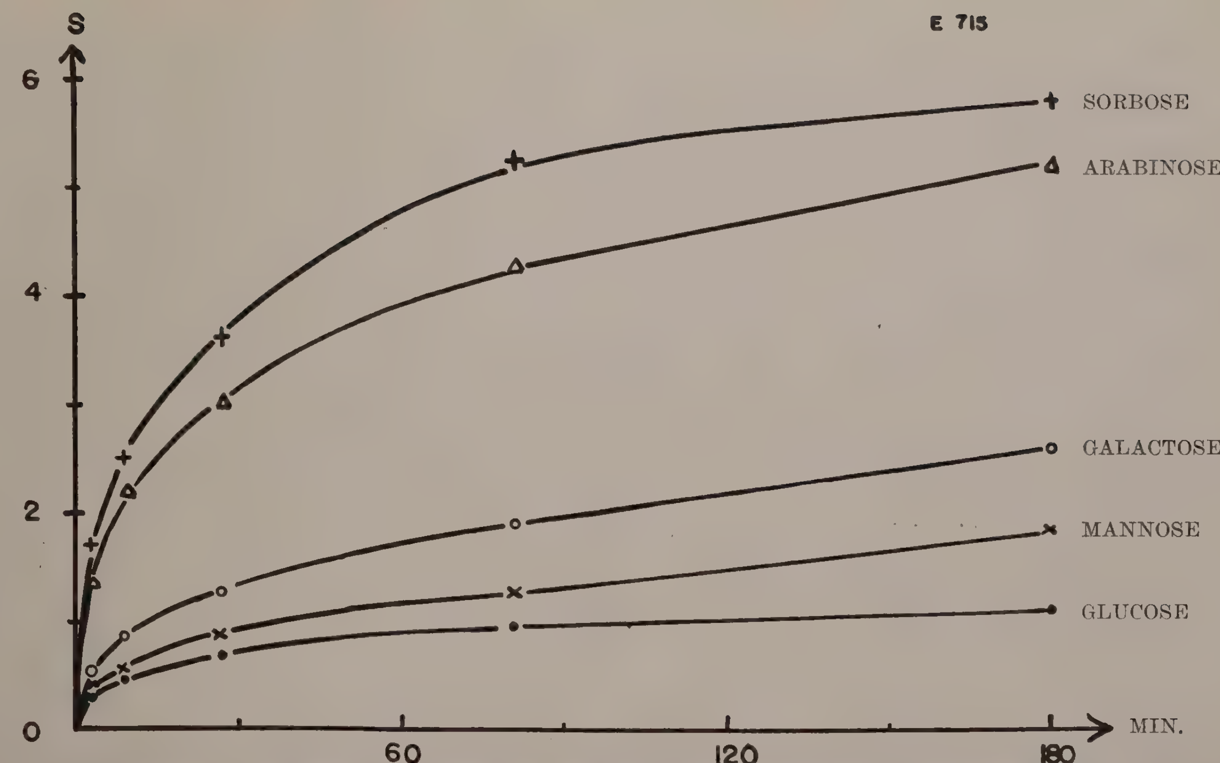

Fig. 2 Penetration of six sugars into human red cells from 1.5 molar solutions (in 0.16 molar NaCl) at 37°C. Indirect method. Ordinate: S = amount of sugar having penetrated the cells (cell units: isotonicity = unit concentration, cell volume = unit volume). P = photometric reading. Abscissa: time in minutes.

its position shifts slightly towards the slower end. Xylose, mannose and glucose in the lowest concentrations used, differ but little in their rates. There remains some uncertainty with respect to their exact order in this concentration range. Possibly in still lower concentrations the order could be estab-

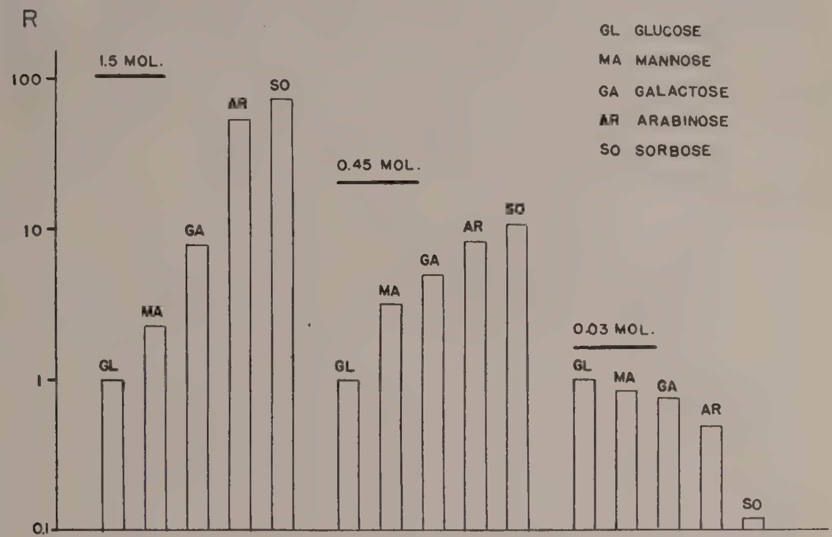

Fig. 3 Relative rates of penetration, R, of six sugars (the rate of glucose arbitrarily taken as unit) from 0.03 molar solutions (direct method), 0.45 molar and 1.5 molar sugar solutions (indirect method). Definition of R: ratio of the penetration times for a given percentage penetration (30%, 80% and 20% from 0.03, 0.45 and 1.5 molar solutions respectively):

$$R = \frac{t_{glucose}}{t_s}$$

($t_{glucose}$ and t_s = times for glucose and the sugar to be compared).

TABLE 1

Order of penetration rates of 7 sugars (fr = fructose, so = sorbose, ar = l-arabinose, ga = galactose, ma = mannose, xy = d-xylose, gl = glucose) in 4 concentrations.

EXPER. NO.	CONCENTR. IN MOL/L	ORDER OF SUGARS
703	0.066	(fr) < so < ar < ga < ma [xy] < gl
705	0.45	gl < (fr) < ma < ga < [xy] < ar < so
704	0.60	gl < ma < (fr) < ga < [xy] < ar < so
715	1.50	gl < ma < ga < (fr) < [xy] < ar < so

lished more clearly, but again from experimental reasons this meets with difficulties.

DISCUSSION

The result of the experiments appears to be in accordance with the predictions derived from the assumed carrier mechanism. With low concentrations the order of penetration rates is roughly that of the affinities as judged from competition experiments. With high concentrations the order is completely reversed for five sugars. The position of two further sugars in the series shifts in the predicted direction as the concentration is raised.

SUMMARY

It is shown that the kinetics of a carrier transport across a membrane should be characterized by a peculiar relation between substrate-carrier-affinity and rate of transport; the rate should be proportional to the affinity, if the substrate concentration is low and inversely proportional, if it is high (relative to the concentration of half saturation).

Experiments on the penetration of sugars across the human red cell membrane qualitatively bear out this prediction. With low concentrations (0.03 molar) the order of penetration rates is glucose > mannose > galactose > arabinose > sorbose. With high concentrations (1.5 molar) the series is reversed: sorbose > arabinose > galactose > mannose > glucose. The position of fructose and xylose in the series also varies with the concentration, in qualitative agreement with prediction.

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POTASSIUM TRANSPORT IN DUCK RED CELLS [1]

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THREE FIGURES

INTRODUCTION

This paper describes the ionic composition and general characteristics of potassium transport in the red blood cells of the duck. Like most (man, rat, rabbit, horse), but not all (dog, cat, beef, certain sheep) mammalian red cells, duck erythrocytes were found to contain much K and little Na, although they are normally suspended in plasma containing much Na and little K. Evidence to be cited below suggests that these large concentration gradients for K and Na also represent large electrochemical activity gradients for both ions across the duck cell surface. Thus, like human red cells (Harris and Maizels, '52; Solomon, '52; Tosteson, '55), duck erythrocytes must continuously do work in order to maintain their normal K and Na composition.

The energy necessary for this work presumably derives from organic reactions occurring within the cell. The mechanism by which such chemical free energy is made available for the transport of K (or for any other kind of transport work) is one of the important unsolved problems of biology. The results described in this paper indicate that the duck red cell

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is a convenient system for study of the coupling of potassium transport with metabolic reactions. For reasons noted below, this is particularly true for duck red cells incubated in nitrogen. Subsequent papers will describe experiments which explore the coupling between metabolic reactions and K transport in this system.

METHODS

Blood was drawn from the jugular vein or heart of adult white male Pekin ducks into heparin (1 mg/10 ml blood) under sterile conditions. Two parts of blood were diluted with three parts of a bicarbonate buffer medium containing 4 mM/l K, 130 mM/l Na and 20 mM/l glucose at pH 7.5 (Tosteson, Carlsen and Dunham, '55). In the experiments in which the K concentration in the medium [$(K)_m$ when expressed in mM/l] was increased, the cells were centrifuged at 800 g, the plasma discarded and the unwashed cells suspended in the above described medium (Tosteson, Carlsen and Dunham, '55) in which some of the NaCl had been replaced by KCl. The resultant cell suspension was incubated in either 95% O_2 — 5% CO_2, 95% N_2 — 5% CO_2, or mixtures thereof, at 37°C. After about one hour of incubation to ensure equilibration with the gas phase, a tracer amount of $K^{42}Cl$ was dumped from a sidearm into the reaction vessel. Aliquots of the cell suspension were removed from the reaction flask at intervals. Part of this aliquot was reserved for analysis of the whole cell suspension. The remainder of the aliquot was immediately centrifuged in special Lucite tubes at 20,000 g in order to obtain samples of medium and cells for analysis.

The centrifuge tubes were so designed that the cells accumulated in a cup of 1 ml capacity separated from a larger chamber at the top of the tube by a narrow neck. After centrifugation, the supernatant medium was removed from the upper chamber, the white cells and a few erythrocytes in the narrow neck discarded, and the red cells removed through a separate opening into the bottom chamber.

The cells were analyzed for K, Na, K^{42} and H_2O, as previously described (Tosteson, Carlsen and Dunham, '55). All cell analyses were corrected for the amount of medium trapped in the packed cells. The trapped medium was found to be $1.0 \pm 0.2\%$ of the packed cell volume. (This measurement was made with C^{14} carboxyl labelled inulin by Dr. E. Cotlove.) When done, chloride measurements on cells and medium were made by a modification of the method of Sendroy (Van Slyke and Hiller, '47). The medium was analyzed for K, Na and K^{42}, whereas the whole cell suspension was analyzed for K, K^{42} and pH (Tosteson, Carlsen and Dunham, '55). Glucose analyses of the whole cell suspension were done by the methods of Miller and Van Slyke ('36), and Nelson ('44), and lactate was measured by the method of Barker and Summerson ('44). Hematocrits were measured in capillary tubes spun at 20,000 g for 10 minutes.

From these data the flux of K across the duck red cell surface was calculated. In most of the experiments, the cell K concentration [$(K)_c$ when expressed in mM/l], did not vary with time and the flux was calculated by the steady state method (Tosteson, Carlsen and Dunham, '55). When $(K)_c$ did vary with time, both influx and outflux were calculated separately by a simplification (Sheppard and Martin, '50) of the previously described unsteady state method (Tosteson, Carlsen and Dunham, '55). The equations used were:

$$^{i}M = \frac{\frac{dX_c}{dt}(K)_c}{X_m - X_c} \qquad (1)$$

$$^{o}M = {}^{i}M - \frac{d(K)_c}{dt} \qquad (2)$$

where ^{i}M and ^{o}M represent influx and outflux expressed in mM/(l RBC.) $\times$ (hr.), X_c and X_m represent cell and medium specific activity, and t is the time in hours. $\frac{d(X)_c}{dt}$ and $\frac{d(K)_c}{dt}$ were obtained graphically.

RESULTS

Ionic composition of cells

When fresh duck red cells were suspended in diluted plasma they maintained K and Na in the steady state (cell concentrations invariant with time) for several hours. The mean cell and medium concentrations of K and Na during this steady state are shown in table 1. The differences between cells incubated in O_2 and N_2 will be discussed in further detail below. Here we wish to point out that duck red cells were found to contain much K and little Na, whereas the diluted plasma in

TABLE 1

Cation and water composition of diluted duck blood

The data in the table were obtained on duck blood diluted with medium incubated at 37° for several hours. The mean of several measurements made on the system during the incubation was recorded. The values in the table represent the mean of these mean measurements from 10 experiments on cells incubated in O_2 and 21 experiments on cells incubated in N_2. The symbol SE indicates the standard error of the mean.

GAS PHASE %	RED CELLS $(K)_c$ mM/l RBC.	$(Na)_c$ mM/l RBC.	H_2O_c gm/gm	DILUTED PLASMA $(K)_m$ mM/l	$(Na)_m$ mM/l	K FLUX mM/(l RBC.) × (hr.)
95 O_2 :	112	7.30	.593	6.06	141	8.0
5 CO_2	SE 1.0	SE 0.97	SE .004	SE 0.44		SE 0.7
95 N_2 :	106	9.04	.645	4.83	145	28
5 CO_2	SE 1.0	SE 0.73	SE .004	SE 0.18		SE 3.5

TABLE 2

Chloride concentration and exchange in duck red cells

The total chloride concentrations in the table represent the mean of two experiments on duck cells incubated at 37°C. in 95% N_2:5% CO_2 at pH = 7.6. The isotopic values are derived from one experiment in which duck cells were incubated for three minutes at 26°C. in 95% N_2:5% CO_2 in a medium tagged with Cl^{38}.

MEAN TOTAL CHLORIDE CONCENTRATIONS			Cl^{38} CONCENTRATIONS AFTER 3 MIN. INCUBATION WITH TRACER		
$[Cl]_c$ mM/kg H_2O	$[Cl]_m$ mM/kg H_2O	$[Cl]_c/[Cl]_m$	$[Cl^{38}]_c$ RCPM/kg H_2O	$[Cl^{38}]_m$ RCPM/kg H_2O	$[Cl^{38}]_c/[Cl^{38}]_m$
82.0	111	.74	.759	1.027	.74

which they were suspended was rich in Na and poor in K. The ratio of cell to medium K concentration (each expressed in mM/kg H_2O) was about 30. In contrast, the ratio of medium to cell chloride concentration (mM/kg H_2O) was about 1.2 at pH 7.6. Cl^{38} was found to reach isotopic equilibrium within three minutes after duck red cells were suspended in a medium containing this tracer (table 2). It is probable that chloride is at thermodynamic equilibrium in this cell system. It follows that K is not at equilibrium. There must be a large electrochemical potential gradient for potassium ions across the plasma membrane of the duck red cell (Harris and Maizels, '52; Solomon, '52; Tosteson, '55).

K transport rates: Effect of gas composition

Cells incubated in O_2. When duck red cells were incubated in 95% O_2 — 5% CO_2 in a medium containing K^{42}, the cell specific activity became equal to that in the medium in less than 24 hours. Thus, there was no evidence for non-exchangeable K. As noted above, during the first several hours of incubation the system remained in the steady state with respect to K. The flux measurements described below were made during this steady state.

Figure 1 shows the cell relative specific activity ($1 - X_c/X_e$ where X_c is the cell specific activity and X_e is the equilibrium or whole cell suspension specific activity) plotted semi-logarithmically as a function of time after addition of K^{42} to a cell suspension incubated in O_2. It is evident that the cell K approached isotopic equilibrium along a curve containing more than one exponential. Curves of this type were obtained in many, but not all, of the experiments with cells incubated in O_2. They indicate that the cell K cannot be considered a single, perfectly mixed compartment under these conditions.

These data were analyzed by assuming that the observed curves could be described by an equation containing two exponential terms. It was also assumed that the cell K comprised two perfectly mixed compartments, either both ex-

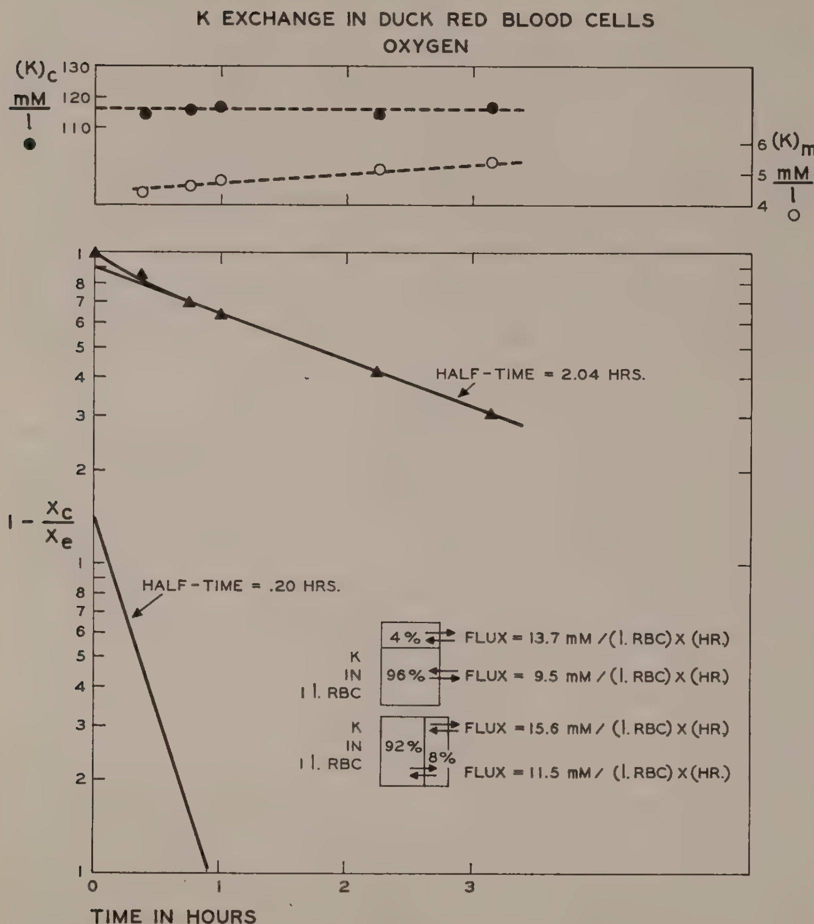

Fig. 1 The graphs show a typical experiment in which diluted duck blood was incubated at 37°C. in 95% O_2:5% CO_2. The upper part of the graph shows the concentration of K in the cells, $(K)_c$, on the left hand ordinate, and in the medium, $(K)_m$, on the right hand ordinate, plotted as a function of time on the abscissa. The lower part of the graph shows the value of $1 - X_c/X_e$, where X_c is cell specific activity and X_e is equilibrium specific activity, plotted on the left hand ordinate as a function of time on the abscissa. The lower line represents the fast component of the exchange curve and was obtained by subtraction of the slow component from the experimental curve. The figures in the inset are calculated fluxes. For details see text.

changing directly with the medium (parallel case) or only one exchanging directly with the medium (series case). The method of calculation of the amount of K in each cell compartment and the fluxes between the compartments from the observed curve for such a three compartment closed system has been previously described (Robertson, Tosteson and Gamble, '54). The results of one such analysis are shown in

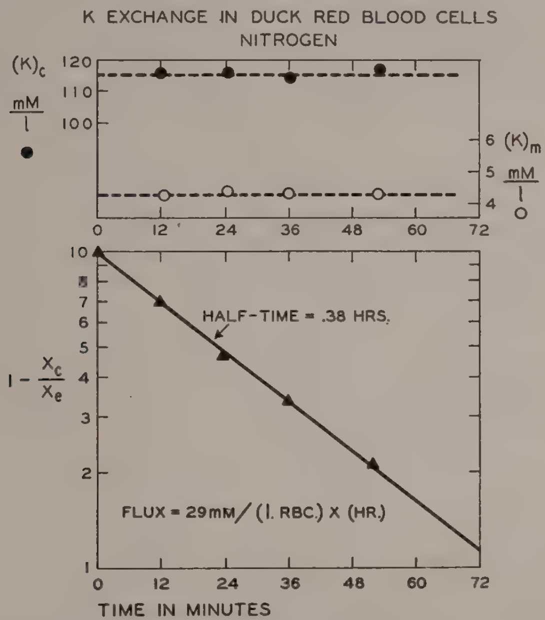

Fig. 2 The graphs show a typical experiment in which diluted duck blood was incubated in 95% N_2:5% CO_2 at 37°C. The ordinates and abscissa are as described in figure 1.

the inset of figure 1. In most of the experiments, the smaller, more rapidly exchanging compartment was too large to be the white cells.

The mean K flux calculated from experiments with duck red cells incubated in diluted plasma at 37°C. in 95% O_2 — 5% CO_2 was 8.0 ± 0.7 mM/(l RBC.) × (hrs.), table 1. In those experiments in which there appeared to be more than one cell K compartment, the flux between the larger cell compartment

and the medium in the "parallel" analysis was used in computing the mean. When the exchange curve followed a single exponential, the calculated two compartment steady state flux was used (Tosteson, Carlsen and Dunham, '55).

Cells incubated in N_2. All of the K in duck cells incubated in 95% N_2 — 5% CO_2 was exchangeable. Upon equilibration with 95% N_2 — 5% CO_2, the water content of the cells increased by 10% (table 1). In association with this increase in volume, the cells gained K so that the concentration of K (per 1 cells) remained virtually unchanged. These adjustments were complete within 15 minutes after removal of O_2.

TABLE 3

Effect of oxygen tension on K fluxes

The values in the table were obtained in a single experiment in which four aliquots of the same diluted duck blood were incubated at 37°C. in 95% N_2:5% CO_2, 85% N_2:10% O_2:5% CO_2, 75% N_2:20% O_2:5% CO_2, and 95% O_2:5% CO_2 respectively. During the incubation period the cells remained in the steady state with respect to K.

OXYGEN TENSION	$(K)_c$	$(K)_m$	H_2O_c	K FLUX
ATM	mM/l		gm/gm	mM/(l RBC.) × (hr.)
0.0	108	11.9	.623	22
0.1	108	13.2	.607	9.4
0.2	108	14.0	.600	8.5
0.95	106	16.0	.588	10

Thereafter the cells maintained K in the steady state for a period of several hours. The flux measurements described below were made during this steady state.

In contrast to the case of duck cells incubated in O_2, the specific activity of K in cells incubated in N_2 approached isotopic equilibrium along a curve which could be described by a single exponential (fig. 2). Under these conditions, cell K behaved as a single perfectly mixed compartment. Furthermore, the mean K flux calculated from such experiments was 28 ± 3.5 mM/(l RBC.) × (hr.), 2–4 times as fast as that observed for cells incubated in O_2 (table 1). In view of these results, experiments were done to define quantitatively the relation between pO_2 and K transport.

Quantitative effect of gas composition on K transport. K transport was twice as fast in cells incubated in 95% N_2 — 5% CO_2 rather than in 85% N_2 — 10% O_2 — 5% CO_2 (table 3). Further increase of the pO_2 to 0.95 atmospheres had no effect

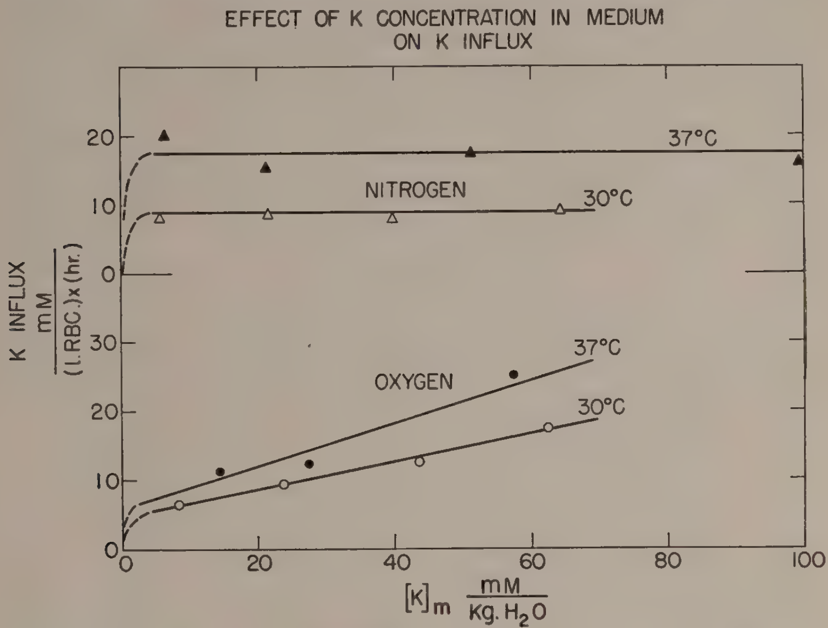

Fig. 3 The upper two graphs show K influx on the ordinate plotted as a function of the K concentrate in the medium, $[K]_m$, on the abscissa for duck red cells incubated in 95% N_2:5% CO_2 at 37°C. and 30°C. The lower two graphs show the relation between K influx and $[K]_m$ for duck cells incubated in 95% O_2:5% CO_2 at 37°C. and 30°C. Each point represents a single experiment.

on the K flux.[3] Acceleration of K transport also occurred when O_2 was replaced by He rather than N_2. Therefore, the acceleration of K transport in N_2 was probably due to hypoxia, not a reversal of an effect of high pO_2, or direct stimulation due to high pN_2.

[3] The flux values for the cells incubated in O_2 in these and subsequently described experiments in this paper are approximate. They were calculated by ignoring the small rapid component(s) frequently present in the exchange curve in the O_2 experiments and drawing the best straight line through the 0, 1 and 2 hour values of $1 - X_c/X_e$. In other words, the flux was calculated assuming that all of the K in cells in O_2 was perfectly mixed.

Effect of varying K concentration in medium on K influx

For duck cells incubated in N_2, K influx became independent of the K concentration in the medium when the latter was above 5–10 mM/kg H_2O (fig. 3). The apparent Michaelis-Menton constant was $1\text{–}3 \times 10^{-3}$ M/l and the maximum influx was 18 mM/(l RBC.) $\times$ (hr.) for cells incubated at 37°C. and 8.4 mM/(l RBC.) $\times$ (hr.) for cells at 30°C.

In contrast, for duck cells incubated in O_2, the relation of K influx, ${}^{i}M$, to the K concentration in the medium ($[K]_m$ when expressed in mM/kg H_2O) was complex (fig. 3). In the region

TABLE 4

Effect of temperature on K flux

The values for K flux at 30°C. shown in the table represent the means of two experiments in 95% O_2:5% CO_2 and three experiments in 95% N_2:5% CO_2. The cells remained in the steady state with respect to K in all of these experiments. The values for K flux at 37°C. are the mean values shown in table 1.

GAS PHASE %	TEMPERATURE °C.	$(K)_c$ mM/l	$(Na)_c$ mM/l	$(K)_m$ mM/l	K FLUX mM/(l RBC.) × (hr.)	Q_{10}
95 O_2:5 CO_2	30	102	6.36	8.14	7.4	1.6
95 O_2:5 CO_2	37	112	7.31	5.85	8.1	
95 N_2:5 CO_2	30	103	5.87	5.98	8.8	4.2
95 N_2:5 CO_2	37	109	10.4	4.64	26	

of the curve between $[K]_m = 0$ and $[K]_m = 10$ mM/kg H_2O, the slope of the line decreased progressively. Above $[K]_m =$ 10 mM/kg H_2O the slope became constant and the curve could be described by the equation ${}^{i}M = 5.8 + 0.30\ [K]_m$, for cells incubated at 37°C. and ${}^{i}M = 4.6 + 0.21\ [K]_m$ for cells at 30°C.

Effect of temperature on K transport

The Q_{10} (30.0–37.0°C.) of K flux was 1.6 for cells incubated in O_2 and 4.2 for cells incubated in N_2 in diluted plasma with $(K)_m \approx 5$ mM/l (table 4). An analysis of the effect of temperature on the relation of K influx to K concentration in the medium will be presented in the discussion.

Effect of pH on K transport

When the pH was lowered from 7.6 to 7.0, there was net transport of K from cells to medium during the one hour period of equilibration of the cells with the gas phase (table 5). During the subsequent two hour experimental period, the oxygenated cells remained approximately in the steady state, while the nitrogen system showed slight net K accumulation. Both influx and outflux were markedly inhibited at the acid pH for cells incubated in N_2. The apparent lack of effect of lowering pH on the K fluxes in the oxygen system may be due to the marked increase in $(K)_m$ which occurred in this experiment.

TABLE 5

Effect of pH on K fluxes

The values in the table were obtained in a single experiment in which four aliquots of the same diluted duck blood were incubated at 37°C. in N_2 and O_2 at pH 7.0 or 7.8.

GAS PHASE	pH	$(K)_c$	$(K)_m$	INFLUX	OUTFLUX
%		mM/l		mM/(l RBC.) × (hr.)	
95 O_2:5 CO_2	7.82	112	5.24	8.2	8.2
95 O_2:5 CO_2	7.02	94.1	12.8	10	10
95 N_2:5 CO_2	7.75	109	3.40	35	35
95 N_2:5 CO_2	7.06	101	6.36	15	13

Effect of composition of medium on K influx

The K flux in duck cells incubated in N_2 was found to be relatively insensitive to various modifications of the medium in which the cells were suspended (table 6). Removal of plasma, removal of Ca, Mg, or both, were all without effect on K flux. Addition of ethylene diamine tetra-acetic acid (versene) to the medium in the absence of Mg and Ca stimu-

lated K influx somewhat. Replacement of the bicarbonate buffer with tris hydroxymethyl amino methane or phosphate did not affect K fluxes in O_2 or N_2.

DISCUSSION

On the basis of these results, it is very probable that the duck red cell incubated under appropriate conditions *in vitro* can maintain a large electro-chemical potential gradient for K ions across its plasma membrane. For example, the ratio of cell to medium K concentration (per kg H_2O) is about 31 for duck cells in N_2. Assuming that the activity coefficient for K is the same inside and outside the cell, and that the electrical potential difference across the cell membrane can be estimated

TABLE 6

Effect of composition of medium on K transport

The table includes flux measurements in single experiments in which the plasma concentration and buffer in the medium were varied, and in which versene was added to the medium. Also included are mean flux measurements from two experiments in which the Ca and Mg concentrations in the medium were varied.

GAS PHASE %	(PLASMA) V/V	BUFFER	$(Mg)_m$ mM/l	$(Ca)_m$ mM/l	$(K)_m$ mM/l	K FLUX mM/(1 RBC.) × (hr.)
95 O_2:5 CO_2	.1	HCO_3	1.00	0.50	9.02	14
95 N_2:5 CO_2	.1	HCO_3	1.00	0.50	6.61	26
100 O_2	.1	Tris	1.00	0.50	9.99	13
100 N_2	.1	Tris	1.00	0.50	7.22	28
100 O_2	.1	Phosphate	1.00	0.50	9.85	17
100 N_2	.1	Phosphate	1.00	0.50	5.77	29
95 N_2:5 CO_2	.1	HCO_3	1.00	0.50	4.61	14
95 N_2:5 CO_2	.01	HCO_3	1.00	0.50	5.90	12
95 N_2:5 CO_2	.01	HCO_3	1.00	0.50	6.49	22
95 N_2:5 CO_2	.01	HCO_3	0.0	0.50	6.09	19
95 N_2:5 CO_2	.01	HCO_3	1.00	0.0	6.22	25
95 N_2:5 CO_2	.01	HCO_3	0.0	0.0	6.13	24
95 N_2:5 CO_2	.01	HCO_3	0.0 Versene	0.0	7.19	33

from the chloride concentration ratio (Solomon, 52; Tosteson, '55), this concentration ratio can be calculated to represent an electrochemical activity ratio of 26.

If K transport in duck red cells were wholly by diffusion, the ratio outflux to influx should equal 26 (Ussing, '49), and the cells would rapidly lose K. In point of fact, the flux ratio was found to be unity (steady state). Thus, K transport in this system involves some process in addition to diffusion. In other words, it is driven by some force in addition to the electro-chemical potential gradient of K. It is reasonable to suppose that this additional force is the electro-chemical potential gradient of some other molecule coupled to the transport of K (Tosteson, '55). For the purpose of discussion, we will refer to K transport involving such coupling as transport by carrier.

Several arguments support the conclusion that K influx involves transport by carrier. (1) Movement of K into the cells is against the electro-chemical potential gradient. (2) K influx into cells incubated in N_2 bears a relation to $[K]_m$ which is characteristic of enzyme reactions and other ''saturateable'' processes. The relation of influx to $[K]_m$ for cells incubated in O_2 is more complicated but still consistent with transport by carrier. (3) The high temperature coefficient of the process is consistent with transport involving some chemical reaction.

For human red cells, it appears possible to account for K transport by assuming that outflux is mostly by diffusion (Tosteson, '55). In contrast, K outflux in duck red cells appears to involve at least some transport by carrier. The two following arguments support this conclusion.

1. If we assume that outflux is wholly by diffusion, we may calculate the rate constant for influx by diffusion from the relation $D'_K = k^o\ [Cl]_m/[Cl]_c$, when D'_K is the inward rate constant for diffusion, k^o is the outward rate constant defined as $^oM/[K]_c$, and $[Cl]_m$ and $[Cl]_c$ represent the chloride concentration per kilogram H_2O in medium and cells respectively

(Tosteson, '55). If we now assume that the total influx, ${}^{i}M$, is equal to the sum of influx by carrier, ${}^{i}M_c$, and influx by diffusion, i.e.

$$ {}^{i}M = {}^{i}M_c + D'_K[K]_m \qquad (3) $$

we can also calculate ${}^{i}M_c$. The values of D'_K and ${}^{i}M_c$, so calculated for cells incubated in N_2 and O_2 at 37°C. and 30°C. are shown in table 7 under the columns headed "flux ratio."

These quantities now may be compared with the values for D'_K obtained from an independent calculation. Here it is assumed that the relation of total K influx, ${}^{i}M$, to the concentration of K in the medium, $[K]_m$, when the latter is above the value necessary to "saturate" the carrier(s) can be described by equation 3. Thus, the slope of the line relating ${}^{i}M$ to $[K]_m$ represents D'_K, and the $[K]_m = 0$ intercept represents ${}^{i}M_c$. The values for D'_K and ${}^{i}M_c$ so obtained are shown in table 7 under the columns headed "concentration curve."

TABLE 7

K influx by diffusion and carrier

The table shows the values obtained for the rate constant for K diffusion, D'_K, and carrier influx, ${}^{i}M_c$, into duck red cells incubated at 30°C., or 37°C. in O_2 or N_2. For details see text.

GAS PHASE	TEMPERATURE	D'_K RATE CONSTANT FOR DIFFUSION				${}^{i}M_c$ CARRIER INFLUX			
%	°C.	kg RBC. H_2O/(1 RBC.) × (hr.)				mM/(1 RBC.) × (hr.)			
		Conc. curve	Q_{10}	Flux ratio	Q_{10}	Conc. curve	Q_{10}	Flux ratio	Q_{10}
95 O_2:5 CO_2	37	.30	2.0	.086	2.3	5.8	1.8	10	2.3
95 O_2:5 CO_2	30	.21		.053		4.6		6.3	
95 N_2:5 CO_2	37	.00		.18	3.8	18	3.1	19	3.5
95 N_2:5 CO_2	30	.00		.067		8.4		7.7	

Agreement between the values of D'_K calculated by the two methods is poor in both cell systems but particularly bad for cells in N_2. Therefore, the assumptions underlying one of the two calculations must be wrong. Further evidence noted below suggests that the most likely source of the discrepancy is

the assumption that K outflux is wholly by diffusion. It is interesting that the temperature coefficients of D'_K and ${}^iM_c$ calculated by both methods considerably exceed values usually observed in free aqueous diffusion. The Q_{10}'s for both quantities are greater in N_2 than in O_2.

2. If duck red cells are allowed to incubate for 24 hours in N_2 in the absence of glucose, K influx is reduced to about 5% of its initial value (Tosteson and Johnson, '56). This occurs when the K concentration in the cells has fallen to only 60–70% of its initial value. Therefore, K outflux must be comparably reduced. It seems unlikely that this reduction in outflux is due to decreased cation diffusion. This conclusion is compatible with the observation that prolonged incubation in the absence of substrate in N_2 does not change the hemolysis time of duck cells suspended in 0.3 M ethylene glycol.

Taken together, both of these arguments suggest that K outflux is not accomplished by diffusion alone. It seems likely that outflux as well as influx involves interaction between K and some carrier molecule(s) for cells incubated in O_2 or N_2. In other words, some process such as exchange diffusion (Ussing, '54) must be involved in K transport in duck red cells. This conclusion is supported by consideration of the thermodynamic efficiency of K transport in this system. If we assume that K influx is a thermodynamically reversible process, and that no energy from K outflux is made available for influx, we may calculate the amount of energy required to maintain K distribution in the steady state. For fresh duck cells incubated in 95% N_2 : 5% CO_2 at 37°C. this amounts to 56 calories/(1 RBC.) × (hr.). Glucose consumption by glycolysis in this system is 1.8 mM/(1 RBC.) × (hr.) (table 8). Krebs et al. have estimated that the change in free energy for conversion of 1 mole glucose to 2 moles of lactate under approximately physiological conditions of pH and concentration is — 49.7 k cal. (Burton and Krebs, '53). Thus, the rate of energy release from glycolysis is 89 cal./(1 RBC.) × (hr.). According to this argument, about 60% of the energy from glycolysis is required for K transport in this system. If, as

is the case in human red cells (Solomon, '52), a similar calculation of the energy required for Na transport yields a figure which is even higher than the K value, the energy requirement for cation transport in duck red cells would exceed the energy supply from glycolysis. Such a calculation must await measurement of the Na fluxes. However, it is possible that the only energy released from glycolysis which is available for work is that stored in the 2 moles of ATP formed per mole of glycose consumed. If we take the free energy of hydrolysis of ATP under approximately physiological conditions to be — 14,000 cal./mol. (Burton and Krebs, '53), [it may be even

TABLE 8

Metabolism of duck red cells

The data in the table are mean values obtained from fresh diluted duck blood incubated at 37°C. for several hours. Included are the results from 9 experiments on cells incubated in oxygen and 17 experiments on cells incubated in nitrogen. The symbol SE indicates the standard error of the mean.

GAS PHASE	GLUCOSE CONSUMPTION	LACTATE PRODUCTION
%	mM/(1 RBC.) × (hr.)	mM/(1 RBC.) × (hr.)
95 O_2:5 CO_2	1.0 SE 0.1	0.1
95 N_2:5 CO_2	1.8 SE 0.2	2.6 SE 0.3

less (Levintow and Meister, '54)], we may calculate that 45 cal./(1 RBC.) × (hr.) is the rate of storage of energy as ATP during glycolysis of duck red cells. Thus, the energy required for K transport is actually greater than the energy made available as ATP in this system. The discrepancy becomes even more striking if one considers the results obtained with NaF (Tosteson and Johnson, '56). In 30 mM/l NaF, the energy required for K transport is 78 cal./(1 RBC.) × (hr.) while the total rate of energy release from glycolysis is less than 50 cal./(1 RBC.) × (hr.). Clearly one or more of the assumptions involved in the efficiency calculation is wrong. The most likely possibility is that some of the energy from K out-

flux is made available for influx. Thus, an appreciable fraction of K transport in duck red cells occurs by a process similar to exchange diffusion.

It is clear that the mechanism of K transport in duck cells in O_2 and N_2 is different. The difference between the two systems with respect to the rate of K transport, the effect of temperature on K fluxes, the effect of $[K]_m$ on K influx, the degree of homogeneity of cell K, all support this conclusion. It seems unlikely that the difference can be explained entirely by a reduced resistance to cation diffusion in the O_2 cell. Rather, it seems probable that there is also a difference in the process by which K transport is coupled to organic chemical reactions in the two systems. This difference may be related to the conversion of duck cell metabolism from respiration to glycolysis when O_2 is removed from the system (table 8). Further work is necessary in order to define the circumstances responsible for the effect of hypoxia on K transport in duck cells.

K influx into duck red cells incubated in N_2 at 37°C. is 28 mM/(l RBC.) $\times$ (hr.) more than 10 times as fast as in human red cells in O_2 or N_2. Duck red cells incubated in N_2 at 37°C. consume 1.8 ± 0.2 mM glucose/hr. and produce 2.6 ± 0.3 mM lactate/hr. (table 8), figures which are about the same as those observed in human red cells (Raker, Taylor, Weller and Hastings, '50). Therefore, K influx per mole of glucose consumed is also more than 10 times faster in duck cells in N_2 than in human cells. Apparently the carrier process assumed to be responsible for K influx is substantially more rapid in the duck cell system in N_2.

Other factors also make this system particularly suitable for the study of interactions between K and hypothetical carrier molecules. In O_2, the duck cells respire, whereas in N_2 they glycolyze (table 8). Since there are fewer organic reactions involved in glycolysis than in respiration, the task of identifying the organic reactions essential for K transport is simplified in the anaerobic system. Finally, the exchange data presented in this paper indicate that all of the K in duck cells

incubated in N_2 behaves as a single perfectly mixed compartment, whereas the K in duck cells in O_2 is kinetically heterogeneous. Flux measurements can be made more easily in the former system.

For these reasons duck red cells incubated in N_2 were chosen as a convenient system in which to explore the relation between organic reactions occurring in the cell and K influx by a presumed carrier process. The results of these experiments will appear in subsequent publications.

We wish to acknowledge the technical assistance of Mrs. Jean Johnson and Mrs. Virginia Muhlberg.

SUMMARY

1. The concentrations of K, Na, Cl and H_2O in duck red blood cells after incubation under various conditions *in vitro* were measured.

2. Duck red blood cells came to chloride isotopic equilibrium in less than three minutes after exposure to a medium containing Cl^{38}.

3. When incubated at 37°C. in a medium containing 140 mM Na/l, 5 mM K/l, glucose as substrate at pH 7.5 with 95% O_2 — 5% CO_2 as gas phase, duck red cells maintained K and Na in the steady state for many hours. All of the cell K was exchangeable under these conditions. However, the approach of the cell specific activity to isotopic equilibrium after addition of K^{42} to the medium could be described by a curve containing at least two exponentials. Therefore, duck cell K under these conditions could not be considered a single, perfectly mixed compartment, but rather was kinetically heterogeneous.

4. When duck red cells were incubated under identical conditions except that the gas phase was 95% N_2 — 5% CO_2, the cells also maintained K and Na in the steady state for many hours, and all of the cell K was exchangeable. The approach of cell specific activity to isotopic equilibrium after addition of K^{42} to the medium usually could be described by a single exponential, so that cell K behaved as a single perfectly mixed compartment. The K flux in duck cells incubated in N_2 was

28 ± 3.5 mM/(l RBC.) $\times$ (hr.), 2–4 times faster than either of the flux values derived from a three compartment analysis of the exchange curve for cells in O_2.

5. The effects of K concentration in the medium, temperature, pH, and the composition of the gas phase and medium on K transport in duck cells are described.

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CONTENTS

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